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MULTI-PARTNER MUTUALISMS: INTERACTIONS AMONG THE MOUNTAIN
PINE BEETLE AND TWO OPHIOSTOMATOID FUNGAL ASSOCIATES

By

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Dissertation

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for the degree of

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in Forestry

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Multi-partner mutualisms: Interactions among the mountain pine beetle and two ophiostomatoid fungal associates

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I investigated interactions between the mountain pine beetle (*Dendroctonus ponderosae*) and its two main ophiostomatoid fungal associates, *Grosmannia clavigera* and *Ophiostoma montium*, as well as interactions between the two fungi. The main research questions were: What drives bark beetle fungal interactions? What is the nature of the interaction between the two species of fungi? I tested the hypothesis that the fungi provide nutritional benefits to the bark beetles. Evidence suggests a nutritional role of fungi in the diet of bark beetles because beetles emerging from attacked trees carrying *G. clavigera* were larger than beetles carrying *O. montium*, which in turn were larger than beetles without either fungus. Larval choice feeding experiments indicate that the two fungi may actually provide complimentary benefits. To address the second question, I tested for competition between the two fungi on artificial media. Growth of each species slowed when it encountered media occupied by the other species, indicating competition; however, both species eventually invaded media occupied by the other species. Although *G. clavigera* colonized unoccupied media the fastest, *O. montium* was more effective in colonizing media occupied by *G. clavigera* when their relative growth rates were considered. In another study, the relative abundances of the two fungi were sampled in beetle-attacked trees in the field over the one year life cycle of the insect. I found no evidence of interference competition, but exploitation competition was prevalent after a year when the fungi co-occurred in the phloem. Finally, I examined whether the two fungi are differentially transported in the mycangia and on the exoskeleton of the beetle using scanning electron microscopy and isolating fungi from the mycangia and elytra. I found no evidence of differential transport of *G. clavigera* or *O. montium* in the mycangia and on the exoskeleton from isolation data. There was also no evidence that one fungus was more likely to be transported on the exoskeleton than the other species using electron microscopy. The fungi appear to exist in the mycangium in an altered, yeast or yeast-like state rather than as conidia.

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Dissertation Overview

This research was conducted in partial fulfillment of the Ph.D. requirements at the University of Montana. This dissertation contains four chapters that were each prepared as distinct papers for publication. Each chapter contains a review of the literature pertinent to its subject area. A brief description of each chapter follows.

Chapter 1: Dietary benefits of fungal associates to an eruptive herbivore: Potential implications of multiple associates on host population dynamics

In this manuscript, I examine the potential nutritional role of *G. clavigera* and *O. montium* to the mountain pine beetle (MPB). I use both field sampling studies and laboratory experiments to investigate the effect of mycophagy by the MPB on beetle performance. The effect of the fungal associates on the nitrogen content of phloem, the diet of the MPB, is also investigated. This manuscript is in press in *Environmental Entomology*.

Chapter 2: Effects of water potential and solute on the growth and interactions of two fungal symbionts of the mountain pine beetle

In this manuscript, I investigate interactions between *G. clavigera* and *O. montium* under controlled conditions in the laboratory. The objectives of this study were to determine the nature of the interaction between *G. clavigera* and *O. montium* and determine the effect of water potential, which changes dramatically in beetle-attacked trees over time, on the growth of each species and on interactions between the fungi.

Chapter 3: Competition and coexistence in a multi-partner mutualism: Interactions between two fungal symbionts of the mountain pine beetle in beetle-attacked trees

In this manuscript, I examine interactions between *G. clavigera* and *O. montium* under natural (field) conditions. Although laboratory experiments allow many variables in the system to be isolated, manipulated, and controlled, the conditions in such studies are highly artificial. This study examines the colonization of the phloem of MPB-attacked trees by the fungi and quantifies the relative prevalence of the two fungi in the phloem over the one-year life cycle of the beetle.

Chapter 4: Transport of microbial symbionts by the mountain pine beetle,

Dendroctonus ponderosae

In this manuscript, I used scanning electron microscopy to examine the bodies and mycangial openings of the MPB. The main aim of the study was to determine if *G. clavigera* and *O. montium* were differentially transported on the body and in the mycangia of the MPB.

Chapter 1

Dietary benefits of fungal associates to an eruptive herbivore: Potential implications of multiple associates on host population dynamics

Abstract

I used the mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins) and its two fungal associates, *Grosmannia clavigera* and *Ophiostoma montium*, to investigate potential nutritional benefits of fungi to bark beetles. I tested for potential effects of feeding on phloem colonized by fungi on beetle performance in field and laboratory studies. The fungi increased nitrogen levels in the phloem of attacked trees by 40%, indicating that it may be an important source of dietary nitrogen for the MPB. However, nitrogen levels of phloem inoculated with fungi in the lab were similar to un-colonized phloem, indicating that the fungi may redistribute nitrogen from the sapwood to the phloem rather than increase absolute levels of nitrogen. Beetles emerging from attacked trees carrying *G. clavigera* were larger than beetles carrying *O. montium*, which in turn were larger than beetles lacking fungi. Results of experimental laboratory studies varied, likely due to differences in the growth and sporulation of fungi under artificial conditions. Results indicate that the two fungi may offer complementary benefits to the MPB because larvae preferentially fed on phloem colonized by both fungi together over phloem colonized by one fungus or un-colonized phloem. Teneral adults pre-emergence fed on spores in pupal chambers when they were produced and consumed little phloem prior to emerging. Teneral adults mined extensively in the phloem before emerging when spores were not produced in the pupal chamber. My results provide evidence for a nutritional role of fungi in the diet of bark beetles and demonstrate that multiple associates may differentially affect beetle performance, which could have important implications for bark beetle population dynamics.

Introduction

Mutualism studies have tended to focus on pairwise interactions; however, many positive interactions involve multiple species (e.g., Stanton 2003, Stachowicz and Whitlatch 2005). A host may have multiple partners that belong to different functional groups that provide a variety of benefits, including nutritional supplementation, transport, protection, and pollination. Hosts may also interact with partners in the same functional group, but little is known about whether such partners provide redundant or complementary benefits (but see Stanton 2003, Stachowicz and Whitlatch 2005).

Many bark beetles (Coleoptera: Curculionidae, Scolytinae) have symbiotic associations with one or more species of fungi in the Ophiostomataceae (Ascomycotina) or their associated anamorphs (asexual states) (reviewed in Six 2003a). Bark beetles inoculate tree tissues with fungi as they construct their galleries and feed in the phloem and inner bark of trees. Bark beetle-fungal interactions have long been assumed to be mutualistic (Craighead 1928). The fungi benefit from consistent dissemination by the beetles to a new, otherwise inaccessible, host tree. In return, it has been hypothesized that the fungi help the beetles kill trees by overwhelming their defenses and interrupting translocation (reviewed in Paine et al. 1997). However, the majority of bark beetle species do not kill trees, despite often having a close association with one or more species of fungi (Six 2003a). The effect of multiple associates on bark beetle performance has only been investigated in a few studies (Goldhammer et al. 1990, Coppedge et al. 1995, Six and Paine 1998), and our understanding of the potential roles of fungal associates, as well as the effect of multiple partners, is limited.

In ambrosia beetles, which are closely related to bark beetles, the nutritional role of fungal associates has been well-demonstrated. Ambrosia beetles inhabit the sapwood of trees, possess mycangia (specialized structures of the exoskeleton for transporting fungi), and feed exclusively on their fungal symbionts which line their galleries (Beaver 1989). A similar nutrition-based symbiosis may also exist with bark beetles because ambrosia beetles are polyphyletic out of bark beetles and ambrosia fungi are derived from lineages of *Ophiostoma sensu lato* and *Ceratocystis* associated with bark beetles (Cassar and Blackwell 1996, Rollins et al. 2001). Unlike ambrosia beetles, bark beetles feed on tree tissues as they construct galleries in the phloem. However, the larvae often consume phloem colonized with fungal hyphae (Adams and Six 2007) and teneral (newly eclosed, sexually immature) adults may feed on thick layers of conidia (asexual spores) that typically line the pupal chambers (Leach et al. 1934, Whitney 1971, Six and Paine 1998). Although phloem is somewhat more nutritious than sapwood, it is still a relatively low quality diet for herbivorous insects. Bark beetles may cope with a low nutrient diet by employing a nutritional supplement in the form of fungi (Ayres et al. 2000).

The mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (MPB) is an eruptive, univoltine bark beetle that causes significant ecological and economic losses in mature (> 60 years) pine forests in western North America (Safranyik and Carroll 2006). Conditions conducive to outbreaks include mild winter temperatures and expansive stands of susceptible host trees. Yet, these conditions do not always result in an outbreak, suggesting that other factors may be involved (Safranyik and Carroll 2006). Fungal symbionts that provide nutritional benefits to the MPB could have important implications for the population dynamics of this eruptive herbivore. The MPB is associated with

numerous microorganisms; however, two ophiostomatoid fungi, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingf. (previously *Ophiostoma clavigerum*) and *Ophiostoma montium* (Rumbold) von Arx, are the only filamentous fungi consistently isolated from its mycangia (e.g., Whitney 1971, Six 2003b). The fungi may be carried on the exoskeleton or in paired mycangia on the maxillary cardines of adults from both sexes (Whitney and Farris 1970). While numerous studies have examined the role of the fungal associates of the MPB in overwhelming tree defenses (e.g., Reid et al. 1967, Raffa and Berryman 1983, Yamaoka et al. 1995), only three studies have considered the potential nutritional benefits of the fungi to the beetle (Nevill and Safranyik 1996, Six and Paine 1998, Bentz and Six 2006). Nevill and Safranyik (1996) examined competitive interactions between *G. clavigera* and a fungal associate of another bark beetle species and observed no effect of *G. clavigera* on MPB survival or development time; however, other fungal species were not excluded from the experiment because wild beetles were used. In contrast, MPB adults associated with a strain of *G. clavigera* isolated from *D. jeffreyi* Hopkins (Jeffrey pine beetle) produced more brood, which developed faster, than insects developing with a strain of *G. clavigera* isolated from a MPB, *O. montium* isolated from a MPB, or a sterile control. However, inferences were limited due to small sample sizes (Six and Paine 1998). Bentz and Six (2006) found that mycelia of *G. clavigera* and *O. montium* contained similar and relatively high levels of ergosterol, a sterol critical for beetle development that insects are not able to synthesize.

In this study, I test the hypothesis that two mycangial fungi (*G. clavigera* and *O. montium*) provide nutritional benefits to their bark beetle hosts. Furthermore, I examine

the potential for differential effects of the two fungi on beetle performance (adult size and development time) and phloem consumption. I predicted that *G. clavigera* provides greater benefits than *O. montium* because *G. clavigera* has a longer shared evolutionary history with the MPB (Six and Paine 1999). Specifically, I address the following questions: Do the fungal associates of the MPB affect beetle performance and phloem consumption? Is feeding on fungi critical for certain life stages (larvae or teneral adults)? Do MPB larvae prefer to feed on phloem colonized by one or both fungal associates or un-colonized phloem? The effect of fungal associates on phloem nitrogen content was also investigated because nitrogen is often the limiting nutritional factor for herbivorous insects (Mattson 1980).

Materials and methods

Effects of fungal associates on beetle performance and phloem consumption

Field study. I isolated fungi from beetles emerging from naturally attacked lodgepole pine trees (*Pinus contorta* var. *latifolia* Dougl. Ex Loud.) in the field to test for the effect of fungal complement on beetle size and emergence time. Isolations were made from beetles collected from two different populations, which were sampled in two different years: DeBorgia, MT (2003) and Butte, MT (2005).

In 2003, I isolated fungi from beetles caught in emergence traps (described below) at three sites located near DeBorgia, MT: Hidden Valley (47°28'35"N and 115°18'21"W; elev. 1005 m), North Divide (47°28'35"N and 115°18'19"W; elev. 1652 m), and South Divide (47°28'44"N and 115°14'51"W; 1532 m). In June, prior to MPB's flight, mesh emergence traps were stapled at 1.3 m above the soil line on the north and south sides of

the bole of five to six trees that had been successfully mass attacked by the MPB in the summer of 2002 at each of the three sites. Each emergence trap covered approximately 1,500 cm² of bark and consisted of a mesh sleeve with a plastic funnel and collection cup at the bottom. Emergence trap captures were collected every three to five days until 28 July when sites were no longer accessible because of wildfires. Emergence was complete at the Hidden Valley site by this time, but was still ongoing at the two higher elevation sites (North and South Divide). I calculated emergence time for each beetle as a proportion of the total emergence period for the tree from which the beetle emerged as follows: (number of days since the first beetle was caught in a trap on that tree)/(total emergence period [i.e., number of days between the first and last beetles to be caught in a trap on that tree]). Beetles were kept in plastic bags with moist paper towels in a refrigerator for up to 5 d until fungal isolations could be made from the mycangia.

I isolated fungi by dissecting both maxillae (containing the mycangia) from the head of up to 20 randomly-selected live beetles per tree per collection date and placing them onto 2% malt extract agar (MEA) amended with 100 ppm cycloheximide (Harrington 1981). Cyclohexamide selects for *Grosmannia* and *Ophiostoma* spp. and helps reduce isolations of ubiquitous, non-symbiotic fungi from the environment (e.g., *Trichoderma* spp., *Penicillium* spp.) that can quickly overgrow cultures. Cultures were grown for a minimum of eight weeks before colonies were identified using morphological characteristics (hyphae, asexual and sexual structures) (Upadhyay 1981). Because some species sporulate more readily on pine twigs than on artificial media, I added pieces of autoclaved pine twigs to the cultures after the initial identifications were made and then

grew the cultures for an additional four to six weeks before re-examining them. The sex of each beetle was determined (Lyon 1958) and pronotal widths measured.

In 2005, I isolated fungi from beetles emerging from short logs that were cut from naturally attacked trees at three sites near Butte, MT: Pigeon Creek (45°49'35"N and 112°23'58"W; elev. 1967 m), Delmoe Lake (45°57'32"N and 112°21'23"W; elev. 2023 m), and American Gulch (46°09'29"N and 112°34'36"W; elev. 1955 m). On 9 July 2005, approximately two weeks before brood adult emergence in the Butte area, one 60 cm long log was cut at 2.0 m above the soil line from the bole of three trees successfully attacked by the MPB in the summer of 2004 at each site (nine trees total). Bolts were placed indoors in opaque rearing cages with transparent collection cups. Collection cups were emptied daily and isolated fungi from the maxillae of up to 12 randomly-selected beetles per cage per day within 24 h of collection. Collections were ceased when cups were empty for more than 5 consecutive days. Emergence time was calculated, the fungi identified, and data recorded as described above.

ANCOVA was used to test how variation in pronotal width of emerging beetles varied among fungal complements. Emergence time was included as a random covariate in the model because beetle size generally decreases over the emergence period (e.g., Safranyik 1976). Because DeBorgia beetles emerged in the field and Butte beetles emerged indoors in rearing cages, which likely affected emergence times, data from the two populations were analyzed separately. In addition to fungal complement and emergence time, the following independent variables were included in the analyses: beetle sex, site, and tree nested within site (random factor). Only beetles with the same fungal complement present in both mycangia were included in the analyses. These

analyses, as well as all other ANOVAs and MANOVAs (listed below), were conducted using a General Linear Model (GLM) approach (JMP® 5.1, SAS Institute, Cary, NC, USA). Significance was declared at $P \leq 0.05$. Significant F tests were followed by Tukey-Kramer's Honestly Significant Difference (HSD) test, which is a conservative post-hoc test appropriate for uneven sample sizes (Sokal and Rohlf 2000).

No choice feeding assays - constant diet. I reared newly hatched, fungus-free MPB larvae to adults in phloem sandwiches (PSs) inoculated with either *G. clavigera*, *O. montium* or neither fungus (control) to determine the effect of diet on development time, adult pronotal width, and gallery area (a surrogate for phloem consumption). Each PS consisted of a 12 x 12 cm square of fresh lodgepole pine phloem placed between two squares of plate glass (adapted from Hansen et al. 2001). The glass was sterilized in 10% bleach (calcium hypochlorite) for 30 minutes and dried prior to use. To obtain fungus-free larvae, MPB eggs were surface sterilized for 4 min in modified White's solution (Barras 1972), rinsed 4 times in sterile water, and then allowed to hatch on sterilized moistened filter paper in a Petri dish. I placed one newly hatched larva into a small notch cut into the center of the phloem square. Eight 4 mm diameter plugs of agar colonized by *G. clavigera*, *O. montium*, or un-colonized MEA (control) were placed at equal distances in a grid across the surface of the phloem; plugs were flattened in the PS and covered most of the surface of the phloem. PSs were held together with rubber bands, sealed with plastic packing tape with holes punched in the sides to allow for air exchange, and stored in plastic containers for the duration of the experiment. Phloem for the experiment was peeled from logs cut from a mature lodgepole pine tree. The rough outer flakes of bark were rubbed off of logs before they were soaked for 15 min in 10% bleach prior to

peeling. The bleach solution did not penetrate the remaining intact bark on the log or contact the phloem surface. Although this method did not eliminate contaminating fungi, it reduced their occurrence. The MPB eggs, lodgepole pine phloem, and isolates of *G. clavigera* and *O. montium* were all collected from the DeBorgia Divide area.

The experiment was conducted in 2004 and repeated in 2005. Both the 2004 and 2005 trials included the following treatment diets: phloem colonized by *G. clavigera* only; phloem colonized by *O. montium* only; and un-colonized phloem. The 2004 trial had an additional treatment diet consisting of phloem colonized by both *G. clavigera* and *O. montium*.

Sandwiches were checked every 4-5 d and the following information was recorded: date of pupation, adult eclosion, and attempted emergence (when adult beetles chewed a hole through to the bark side of the PS). Any mortality was recorded. The date of these events was recorded as the half point of the preceding observation period. The presence of any conidia lining the pupal chambers was noted. Upon attempted adult emergence, PSs were disassembled and the sex and pronotal width of each beetle was recorded. The outlines of both larval and teneral adult galleries were traced onto clear plastic sheets, scanned, and the number of pixels in each area calculated using Adobe® Photoshop® v. 9 (Adobe Systems Inc., San Jose, California, USA). To convert to cm², the number of pixels in each gallery area was divided by the number of pixels in 1 cm². I also calculated gallery volume for a subsample of PSs by making a paraffin wax mold of the galleries and measuring the amount of water each wax mold displaced.

Visual inspection of PSs indicated that *G. clavigera* and *O. montium* had successfully colonized the phloem in sandwiches receiving fungal treatments, but not in the un-

colonized controls. Treatment diets were also confirmed as successful by identifying conidia present in pupal chambers and by re-isolating fungi from phloem adjacent to pupal chambers.

Data from the 2004 and 2005 trials were analyzed separately because different treatment diets were used in each year. I used MANOVA to test for an effect of treatment diet on development time (number of days as a larva, pupa, and teneral adult and total development time [from neonate larva to attempted adult emergence]) as well as on gallery area (larval, teneral adult, and total). Significant MANOVAs were followed by ANOVAs to determine which response variables contributed to the treatment differences. The strength of the relationship between gallery area and gallery volume was tested using Pearson product-moment correlations. Because gallery area was highly correlated with gallery volume, area was used in all subsequent analyses. ANOVA was used to test for variation in adult pronotal width among treatments. Sex and sex by treatment diet interaction terms were included in all models. Differences among the treatment diets in the presence/absence of conidia in the pupal chamber were analyzed using the Likelihood Ratio Chi-square test (also known as the *G*-test) (Sokal and Rohlf 2000).

Importance of fungal associates to larvae versus teneral adults

No choice feeding assay - alternating diets. To determine if feeding on fungi was more critical to larvae or to teneral adults, newly hatched, fungus-free larvae were placed in PSs (as described above) and reared with either *O. montium* (M) or a MEA control (U) until adult eclosion. *G. clavigera* was not used in this experiment because it did not readily sporulate in pupal chambers in PSs. PSs were monitored daily. Immediately upon eclosion, I switched each teneral adult with a teneral adult from a PS that had either the

other treatment diet or the same treatment diet (to control for the effects of handling). This resulted in four diet combinations (larval diet/teneral diet): U/U, U/M, M/U, and M/M. The number of beetles successfully reared through each treatment was 8, 8, 9 and 6, respectively. The presence of conidia in pupal chambers at the time of switching was noted. The experiment was conducted once in 2004 using eggs, phloem, and *O. montium* (same isolate used in the constant diet assay) collected from the DeBorgia Divide area.

ANOVA was used to test for the following: (1) variation in larval development time with larval diet (i.e., treatment diets U/U, U/M vs. M/M, M/U); (2) variation in teneral adult feeding time with teneral adult diet (i.e., U/U, M/U vs. M/M, U/M); (3) variation in total development time with combined diet (i.e., U/U vs. U/M vs. M/M vs. M.U); (4) variation in larval gallery area with larval diet (as in 1); (5) variation in teneral adult gallery area with teneral adult diet (as in 2); (6) variation in total gallery area (larval and teneral adult) with treatment diet (as in 3); and (7) variation in pronotal width with larval diet because adult size is determined prior to feeding by teneral adults (as in 1). Sex and sex by treatment interaction terms were included in all models.

Feeding preference of the MPB

Choice feeding assay. Choice feeding assays were conducted using PSs to determine the feeding preference of MPB larvae. I offered wild, late instar (3rd or 4th) larvae pairwise choices of lodgepole pine phloem colonized with: *G. clavigera* (C), *O. montium* (M), both *G. clavigera* and *O. montium* (CM), or un-colonized phloem (U) (control). To prepare the phloem used in the assay, PSs containing 12 x 12 cm squares of lodgepole pine phloem were inoculated with eight 5 mm diameter plugs of MEA colonized by fungi or sterile MEA (un-colonized control) as described above. PSs were held for 21-28 d to

allow the fungi to fully colonize the phloem, after which time it was cut into 4 x 4 cm squares with a notch incised in the middle of one side. Two squares of phloem were placed adjacent to one another in a new PS and one wild larva was placed in the 8 x 4 mm chamber made by aligning the notches. Larvae that had mined at least 3 mm into one of the phloem squares after 24 h were considered to have made a choice and only larvae that made a 'choice' were included in the analyses. In order to determine if larval feeding preference in the assay was affected by past feeding experience, a small piece of phloem and frass adjacent to where each larva was found under the bark was placed onto cycloheximide-amended MEA. Isolated fungi were identified as described above.

The experiment was conducted twice using wild larvae from the DeBorgia and Butte populations. *Grosmannia clavigera* and *O. montium* used in the DeBorgia and Butte trials were isolated from beetles taken from their respective populations. The following pairwise contrasts were tested in both trials: C-U, M-U, C-M, C-CM, and M-CM. One additional comparison was made in the Butte trial: U-CM. A sample of phloem from 5 PSs per treatment diet in the DeBorgia trial and from 13 PSs per treatment diet in the Butte trial were frozen (-80°C) for subsequent nitrogen analysis (described below).

Two-tailed exact binomial tests with a hypothesized proportion of 0.5 (i.e., H_0 : no preference) were conducted on the pair wise choices using S-PLUS® 7 (Insightful Corp., Seattle, WA, USA). No statistical tests were conducted on the potential effects of prior feeding experience in the tree on feeding preference in assays because very few larvae were associated with only one fungus in the tree (most were associated with both fungi) (see Results).

Effects of the fungal associates on phloem nitrogen

I determined total nitrogen (percent dry weight) for the phloem used in the choice assay PSs (described above) as well as for phloem samples taken from naturally attacked trees and un-attacked (healthy) trees of similar diameter. On 7 September 2006, three phloem samples were taken from around the circumference of the bole at 1.3 m above the soil line from 15 un-attacked trees, 15 trees mass attacked by the MPB eight weeks prior to sampling, and 9 trees mass attacked by the MPB two weeks prior to sampling. The eight-week old MPB attacks were attacked during the beetle's main flight in the area (late July) and trees contained late instar larvae with phloem that was completely discolored indicating extensive colonization by fungi. The two-week old attacks were attacked during a smaller second flight (late August) and parent beetles were actively elongating galleries and laying eggs in these trees; the only discolored phloem occurred within a few mm of the egg galleries. All trees were located within 1.5 km of each other and were close to the North site (DeBorgia Divide) described previously. Phloem samples were taken adjacent to egg galleries on attacked trees and care was taken to exclude any insects and their frass from the sample. A small piece of phloem from each sample was placed onto MEA and the isolated fungi identified as described previously. Phloem samples were oven dried and then homogenized using a SPEX CertiPrep Geno/Grinder[®] 2000 (Fisher, Pittsburgh, PA, USA) before total nitrogen was determined in an elemental analyzer (EA 1110, CE Elantech, Inc., Lakewood, NJ, USA).

Total nitrogen content (percent dry weight) of a subset of MPBs sampled in the field study was also determined. Only beetles from the Butte population were used because they had been frozen shortly after emergence. Nitrogen content was determined (for each

individual beetle) for 14 randomly selected beetles (6-8 of each sex) that emerged carrying *G. clavigera* only, *O. montium* only, and the six beetles that emerged without any ophiostomatoid fungi in the elemental analyzer as described above.

The effect of treatment diet on the nitrogen content of phloem used in the choice feeding preference assay was tested using one-way ANOVAs. I analyzed data from the DeBorgia and Butte trials separately because the phloem used in the two trials came from two different trees. ANOVA was also used to test for an effect of attack status of trees sampled in the field (trees attacked eight and two weeks prior to sampling and un-attacked, healthy trees) on nitrogen content. Tree nested under attack status was included as a random factor to account for between tree variations in nitrogen. The effect of fungal complement on whole beetle nitrogen was assessed using an ANOVA. Because both fungi were often isolated from the phloem samples taken from attacked trees, the effect of fungal species (*G. clavigera* vs. *O. montium*) on phloem nitrogen could not be tested.

Results

Effects of fungal associates on beetle performance and phloem consumption

Field study. Peak emergence (number of days after the first beetle emerged from that site or population) in the DeBorgia population occurred around day 14 at Hidden Valley and day 30 for the North and South Divide sites. Peak emergence occurred around days 4 and 5 for the Butte sites; however, the emergence period was likely condensed for the Butte beetles because they were collected from logs stored indoors under warmer and more constant temperatures. The proportion of beetles emerging with different fungal

complements did not show any discernable pattern over the emergence period that would indicate a relationship between fungal complement and emergence time (Fig. 1).

Mean pronotal width of beetles emerging from the DeBorgia population varied with fungal complement ($F_{3,563} = 22.56$, $P < 0.0001$) and sex ($F_{1,563} = 149.80$, $P < 0.0001$), but not site ($F_{2,14} = 0.36$, $P < 0.70$). Differences among trees and emergence time accounted for 7% and 23% of the total variation, respectively. DeBorgia beetles emerging carrying *G. clavigera* only were larger than beetles carrying *O. montium* only, which in turn were larger than beetles emerging without either fungus ($P \leq 0.05$) (Fig. 2). Mean pronotal width of DeBorgia beetles carrying both fungi was not different from beetles carrying only one fungal associate ($P > 0.05$) (Fig. 2). Similarly, mean pronotal width of beetles emerging from the Butte population also varied with fungal complement ($F_{3,217} = 9.12$, $P < 0.0001$) and sex ($F_{1,217} = 101.59$, $P < 0.0001$), but not site ($F_{2,6} = 0.55$, $P = 0.60$). Differences among trees and emergence time accounted for 5% and 45% of the total variation, respectively. Butte beetles emerging carrying *G. clavigera* only or both fungi were larger than beetles carrying *O. montium* only, which in turn were larger than beetles emerging without either fungus ($P \leq 0.05$) (Fig. 2). Female beetles were larger than males in both populations ($P \leq 0.05$).

No choice feeding assay – constant diet. Mortality was very high across all treatment diets and the leading cause of death was probably due to handling effects on delicate neonate larvae because many insects failed to enter the phloem after introduction into PSs. Many late instar larvae and pupae also died when they were overgrown by contaminants (mostly *Penicillium* or *Rhizopus* spp.) that invaded some of the PSs. Due to

the high mortality associated with non-treatment effects, survival was not included as an indicator of beetle performance in the analysis.

In the 2004 trial, development times (larval, pupal, teneral adult, and total) did not vary with treatment diet ($\lambda_{12,151} = 0.72$, $P = 0.08$), sex ($F_{4,57} = 0.10$, $P = 0.23$), or the sex by treatment diet interaction ($\lambda_{12,151} = 0.82$, $P = 0.44$). However, in the 2005 trial, development times varied with treatment diet ($\lambda_{8,166} = 0.67$, $P < 0.0001$) and sex ($F_{4,83} = 0.10$, $P = 0.05$), but not their interaction ($\lambda_{8,166} = 0.88$, $P = 0.24$). In 2005, mean larval development time for insects developing with *O. montium* was longer than for insects developing with *G. clavigera* or on un-colonized phloem ($F_{2,86} = 17.08$, $P < 0.0001$). Females spent an additional 3.5 d as larvae compared to males ($F_{1,86} = 10.03$, $P = 0.002$). The number of days insects spent as teneral adults did not contribute to variation among the treatment diets ($F_{2,86} = 2.66$, $P = 0.08$) or between the sexes ($F_{1,86} = 0.01$, $P = 0.91$). Mean total development time for insects developing on un-colonized phloem was shorter than for insects developing with *G. clavigera* or *O. montium* ($F_{2,86} = 8.34$, $P = 0.0005$).

Larval gallery area, teneral adult gallery area, and total gallery area were well-correlated with their corresponding gallery volumes ($r = 0.71$, $r = 0.92$, and $r = 0.84$, respectively, with $P < 0.0001$ for all correlations). Thus, gallery area was used as a surrogate for the amount of phloem ingested. In 2004, gallery areas varied with treatment diet ($\lambda_{9,141} = 0.47$, $P < 0.0001$), sex ($F_{3,58} = 0.40$, $P = 0.0002$), but not their interaction ($\lambda_{9,141} = 0.90$, $P = 0.71$). Larval gallery area, teneral adult gallery area, and total gallery area each varied with treatment diet ($F_{3,141} = 4.93$, $P = 0.004$; $F_{3,141} = 14.04$, $P = 0.004$; $F_{3,141} = 17.26$, $P = 0.0001$, respectively). In 2004, larvae feeding on *G. clavigera*-colonized or un-colonized phloem consumed more than larvae feeding on phloem

colonized by both fungi ($P \leq 0.05$). The amount of phloem consumed by larvae feeding on the *O. montium* treatment diet did not differ from any of the other diets ($P > 0.05$). Teneral adults developing on *G. clavigera*-colonized phloem consumed more phloem than insects developing with both fungi together or *O. montium* only ($P \leq 0.05$). Insects developing with *G. clavigera* only consumed similar amounts of phloem as insects developing with un-colonized phloem or phloem colonized by both fungi together ($P > 0.05$); however, they consumed more phloem than insects developing with *O. montium* only ($P \leq 0.05$). These trends led to total gallery area being greater for insects developing on *G. clavigera*-colonized or un-colonized phloem compared to insects developing on treatment diets with *O. montium* present ($P \leq 0.05$). Females had larger larval gallery areas, teneral adult gallery areas, and total gallery areas than males ($F_{3,58} = 13.75$, $P = 0.0005$, $F_{3,58} = 7.04$, $P = 0.01$, $F_{3,58} = 23.23$, $P < 0.0001$, respectively).

In the 2005 trial, gallery areas (larval and teneral adult) did not vary with treatment diet ($\lambda_{6,168} = 0.28$, $P = 0.28$), but did with sex ($F_{3,84} = 8.69$, $P < 0.0001$). Females had greater larval gallery areas and total gallery areas than males, but teneral adult gallery areas did not differ ($F_{1,86} = 26.53$, $P < 0.0001$, $F_{1,86} = 14.62$, $P = 0.0002$, $F_{1,86} = 0.84$, $P = 0.36$, respectively). There was no interaction between sex and diet ($\lambda_{6,168} = 0.97$, $P = 0.37$).

In the 2004 trial, pronotal width varied with treatment diet ($F_{3,60} = 5.90$, $P = 0.001$), sex ($F_{1,59} = 42.77$, $P < 0.0001$), but not their interaction ($F_{3,60} = 0.97$, $P = 0.41$). Insects developing on un-colonized phloem were larger than insects developing on treatment diets with *G. clavigera* present (C and CM) ($P \leq 0.05$). Insects developing with *O. montium* were larger than insects developing with both fungi ($P \leq 0.05$). In the 2005 trial,

pronotal width also varied with treatment diet ($F_{2,86} = 16.42$, $P < 0.0001$), sex ($F_{1,86} = 35.73$, $P < 0.0001$), but not their interaction ($F_{2,86} = 2.07$, $P = 0.13$). Insects developing on un-colonized phloem were larger than insects developing with *G. clavigera* only or *O. montium* only ($P \leq 0.05$). Females were also larger than males in both trials.

Conidia were produced in pupal chambers more often in treatment diets with *O. montium* present than in treatment diets with *G. clavigera* only in the 2004 trial ($\chi^2_{2,40} = 9.35$, $P = 0.009$) (Table 1). In the 2005 trial, production of conidia in pupal chambers was extremely low and the proportion of pupal chambers with conidia present did not differ between the *G. clavigera* and *O. montium* treatment diets ($\chi^2_{1,38} = 2.65$, $P = 0.10$) (Table 1).

Importance of fungal associates to larvae versus teneral adults

No choice feeding assay - alternating diets. Larval development time did not vary with treatment diet ($F_{1,30} = 1.615$, $P = 0.214$), sex ($F_{1,30} = 2.055$, $P = 0.163$), or their interaction ($F_{1,30} = 0.067$, $P = 0.797$). Similarly, teneral adult development time did not vary with teneral adult treatment diet ($F_{1,30} = 3.296$, $P = 0.080$), sex ($F_{1,30} = 0.167$, $P = 0.685$), or their interaction ($F_{1,30} = 0.226$, $P = 0.637$). Total development time did not vary among the four treatment diets ($F_{3,30} = 1.063$, $P = 0.383$), or between the sexes ($F_{1,30} = 1.935$, $P = 0.177$), or their interaction ($F_{3,30} = 0.823$, $P = 0.494$).

Larval gallery area varied with treatment diet ($F_{1,30} = 17.30$, $P = 0.0003$). Larvae developing on un-colonized phloem (U/U and U/M treatment diets) consumed more phloem than larvae feeding on phloem colonized by *O. montium* (M/M and M/U treatment diets) ($P \leq 0.05$) (Fig. 3). Female larvae consumed more phloem than males ($F_{1,30} = 6.44$, $P = 0.017$) and there was no interaction between sex and larval treatment

diet ($F_{1,30} = 0.105$, $P = 0.749$). Teneral adult gallery area also varied with treatment diet ($F_{1,30} = 8.394$, $P = 0.007$). Teneral adults feeding on un-colonized phloem consumed almost three times as much phloem as teneral adults maturation feeding on *O. montium*-colonized phloem ($P \leq 0.05$) (Fig. 3). Conidia were common in the pupal chambers of 9 of the 14 PSs where teneral adults fed on *O. montium*-colonized phloem. Teneral adults in chambers with conidia present almost always fed on them before attempting to emerge directly from the chamber resulting in no, or very low, teneral adult gallery areas. Teneral adults in pupal chambers without spores usually mined the phloem before attempting to emerge some distance from the chamber resulting in greater teneral adult gallery areas. There were no differences in teneral adult gallery areas between the sexes ($F_{1,30} = 1.435$, $P = 0.241$), and there was no sex by teneral adult treatment diet interaction ($F_{1,30} = 0.219$, $P = 0.643$). The consumption differences resulted in differences in total gallery area among the four treatment diets ($F_{3,30} = 5.402$, $P = 0.006$). Total gallery area of insects feeding on un-colonized phloem as larvae (U/M and U/U) was greater than for insects feeding on *O. montium*-colonized phloem as both larvae and adults (M/M) ($P \leq 0.05$) (Fig. 3). Total gallery area of insects feeding on *O. montium*-colonized phloem as larvae and un-colonized phloem as teneral adults (M/U) was not different than for insects on any other treatment diet ($P > 0.05$) (Fig. 3). Females consumed more phloem than males ($F_{1,30} = 6.979$, $P = 0.015$) but the sex by treatment interaction was not significant ($F_{3,30} = 2.702$, $P = 0.069$).

Pronotal width varied with larval treatment diet ($F_{1,30} = 14.525$, $P = 0.0007$), sex ($F_{1,30} = 32.281$, $P < 0.0001$), but not with the larval treatment by sex interaction ($F_{1,30} = 0.003$, $P = 0.955$). Adult beetles eclosing from larvae that fed on un-colonized phloem

were larger than adult beetles eclosing from larvae that fed on *O. montium*-colonized phloem ($P \leq 0.05$) (LS means (\pm SE) 1.83 (\pm 0.03) mm and 1.68 (\pm 0.03) mm, respectively). Females were larger than males.

Feeding preference of the MPB

Choice feeding assay. In the DeBorgia trial, 257 out of the 269 larvae used in the experiment were associated with both *G. clavigera* and *O. montium* in the tree from which they were collected. Four larvae were associated with *G. clavigera* only whereas eight larvae were associated with *O. montium* only. In the Butte trial, 175 out of 271 larvae were associated with both fungi in the tree from which they were collected, while 61 were associated with *O. clavigerum* only and 35 with *O. montium* only. The effect of prior fungal association on feeding preference in the assay was not statistically testable because most insects were associated with both fungi. However, in the Butte trial, where multiple insects with only one fungal associate were assigned to the same choice test, larval feeding preference appeared to follow the same pattern as insects associated with both fungi that were given the same choice test.

MPB larvae from the DeBorgia population exhibited a strong preference for phloem colonized by *G. clavigera* only or *O. montium* only over un-colonized phloem ($P \leq 0.007$); however, there was no predilection for one fungus over the other ($P = 0.22$) (Fig. 4). In contrast to the DeBorgia trial, larvae from the Butte population exhibited no preference for phloem colonized by *G. clavigera* only or *O. montium* only over un-colonized phloem ($P > 0.07$); however, phloem colonized by *G. clavigera* only was preferred over phloem colonized by *O. montium* only ($P < 0.001$) (Fig. 4). Larvae from both populations preferred phloem colonized by the two fungi together compared to any

other choice ($P < 0.03$), with the exception of the CM-M contrast, which was not significantly different for larvae from the DeBorgia population ($P = 0.21$) (Fig. 4).

Effects of the fungal associates on nitrogen content

Phloem sandwiches. Nitrogen content of phloem used in sandwiches in the choice feeding assay did not vary with treatment diet in either the DeBorgia trial ($F_{2,12} = 0.61$, $P = 0.56$) or Butte trial ($F_{3,48} = 1.67$, $P = 0.19$). Mean nitrogen content of the treatment diets ranged between 20 and 22% in the DeBorgia trial and 25 and 27% in the Butte trial.

Naturally attacked trees. *Grosmannia clavigera* and/or *O. montium* were always isolated from the thin strip of discolored phloem directly adjacent to egg galleries in trees with two-week old MPB attacks; no fungi were isolated from the pink, non-discolored phloem of trees with two-week old attacks. *Grosmannia clavigera* and/or *O. montium* were always isolated from the phloem of trees with eight-week old MPB attacks and all the phloem was completely discolored in these trees. No fungi were isolated from un-attacked trees. The effect of fungal species (*G. clavigera* versus *O. montium*) on phloem nitrogen was not statistically testable because of the limited number of samples from which only one species of fungus was isolated. However, phloem nitrogen was higher in trees with eight-week old MPB attacks than in trees with two-week old attacks or in un-attacked trees (Fig. 5).

Adult MPBs. The fungal complement of emerging beetles had no effect on insect nitrogen content ($F_{2,31} = 1.15$, $P = 0.33$). Mean (\pm SE) nitrogen levels of adult MPBs carrying *G. clavigera* only, *O. montium* only, and neither fungus was 10.2% (± 0.23), 9.8% (± 0.23), and 9.6% (± 0.35), respectively.

Discussion

The MPB lives in a relatively nutritionally poor habitat. Although the beetle avoids interspecific competition by attacking living trees, brood developing at the high attack densities required to kill living trees may be adversely affected by intraspecific competition. A nutritional supplement could potentially benefit bark beetles by increasing diet quality and alleviating intraspecific competition for insects developing under crowded conditions. I examined the potential for the MPB to gain nutritional benefits from its fungal associates.

The fungal associates of the MPB may increase dietary nitrogen in the phloem during the period when mid-to-late instar larvae are feeding (Fig. 5). The fungal associates of the MPB increased phloem nitrogen by 40% eight weeks after attack (from 0.27% to 0.38%). Although MPBs, which are composed of approximately 10% nitrogen, likely still face a significant challenge obtaining sufficient nitrogen in fungal-colonized phloem, even relatively small increases in nitrogen in low quality diets may have significant effects on insect performance (Mattson 1980). Ayres *et al.* (2000) also found that nitrogen levels were higher in phloem colonized by the fungal associates of *D. frontalis* Zimmermann than in patches of un-colonized phloem found in attacked trees. In that study, as well as the current study, the higher nitrogen levels of fungal-colonized phloem compared to un-colonized phloem may be due to higher nitrogen levels in fungal hyphae compared to tree phloem (Ayres et al. 2000). An increase in phloem nitrogen due to the (induced) wound response that trees produce in response to beetle attack and fungal inoculation can be ruled out because nitrogen was not elevated in trees with two-week old MPB attacks and the induced response occurs within days of attack in lodgepole pine (Raffa and Berryman

1982). Furthermore, nitrogen levels only differed between colonized and un-colonized phloem in trees and not between colonized and un-colonized phloem in PSs, which suggests that the fungi may be acquiring and concentrating nitrogen from the sapwood. Fungi are capable of redistributing nutrients within an individual thallus (the vegetative body of a fungus). In attacked trees, fungi may be transporting nitrogen acquired by hyphae invading the sapwood to hyphae growing in the phloem to support the production of thick layers of spores that are produced in the cavities made by the bark beetles (pupal chambers and old galleries). Beetle larvae may benefit from the increase in nitrogen in the phloem tissue where it feeds.

Increases in dietary nitrogen associated with fungal-colonized phloem may explain why beetles that emerged from attacked trees carrying *G. clavigera* and/or *O. montium* were larger than beetles emerging without these fungi (Fig. 2). The greater size benefit conferred by *G. clavigera* compared to *O. montium*, indicates that *G. clavigera* may be more efficient in concentrating nitrogen. Insect size has also been positively correlated with dietary nitrogen for species that feed on diets low in nitrogen (e.g., Fox and Macauley 1977, Mattson 1980, Ayres et al. 2000), but not for species that feed on higher nitrogen diets (Slansky and Feeny 1977). Despite diets with apparently different nitrogen contents, nitrogen levels did not vary among beetles emerging with different fungal complements. Insects may regulate body nitrogen maintaining homeostasis in elemental composition (e.g., Fox and Macauley 1977, Slansky and Feeny 1977), and instead cope with low quality diets by delaying development time, increasing consumption (compensatory feeding), or employing a dietary supplement (Mattson 1980). There is no evidence that the MPB copes with lower quality diets with longer development times

because emergence times did not vary with fungal complement (Fig. 1). Development time may be controlled by other selective forces, such as temperature-driven developmental synchrony, which favors simultaneous emergence essential for coordinating successful mass attacks (Bentz et al. 1991, Jenkins et al. 2001). It is likely that the fungi supplement the diet of the MPB by increasing nitrogen, although it is not possible to rule out the potential effects of compensatory feeding because consumption levels were not measurable in the field study.

In contrast to the field study and my prediction, phloem colonized by *G. clavigera* appeared to be a poor diet for the MPB in the no choice feeding assay in both years. Despite high consumption relative to the other treatment diets, adult beetle size was reduced on *G. clavigera*-colonized phloem. Surprisingly, results for the other treatment diets varied between trials with *O. montium* being the superior diet in 2004 and uncolonized phloem the superior diet in 2005 based on beetle size for the amount of phloem consumed. Six and Paine (1998) also had contrasting results when they experimentally manipulated the fungal complement of MPBs and introduced them into short logs. The MPB had shorter development times and higher brood production when it developed with *G. clavigera* isolated from *D. jeffreyi* (a sister species to the MPB) compared to beetles developing with *O. montium* and or no fungi. However, no progeny were produced by beetles carrying *G. clavigera* isolated from a MPB and the weight of emerging beetles did not differ among the treatments independent of sex. Thus, even different isolates of the same species of fungus may have variable effects on the MPB; however, non-treatment related effects (e.g., mating success, handling effects) may have also affected results because of low sample sizes (Six and Paine 1998). The contrasting results

obtained in this study may, at least in part, be attributed to differences in fungal growth between the 2004 and 2005 trials, which was likely a result of variable environmental conditions (e.g., lab temperature or humidity, phloem chemistry or moisture content, fungal contaminants). Although the same fungal isolates were used in both trials, *O. montium* readily produced conidia in PSs in 2004, but not in 2005, and *G. clavigera* rarely sporulated in pupal chambers regardless of trial (Table 1).

The contradictory field and laboratory results may be due to the artificial conditions in PSs, which are dramatically different than the conditions the fungi would experience in naturally attacked trees over the one year life cycle of the insect. For example, in PSs fungal hyphae are only able to absorb nitrogen from the same piece of phloem as the insects consume. Thus, any increase in nitrogen in the hyphae would be counter balanced by a reduction in nitrogen in the adjacent phloem, resulting in no dietary benefit (in terms of nitrogen) to the larva as it mines the phloem. If fungi rely on nitrogen inputs from the sapwood this could affect spore production in PSs. Both fungi are commonly found sporulating in pupal chambers in naturally attacked trees, but sporulation was highly variable in PSs. This hypothesis would also explain why nitrogen content of phloem from the same tree, but inoculated with different treatment diets, did not vary. Although the results of the no choice feeding assay constant diet must be interpreted with caution given the artificial conditions in PSs, the experiment highlights the potential effects of variation in environmental conditions and fungal sporulation on bark beetle-fungal interactions.

Larvae from DeBorgia and Butte populations differed in their preferences for uncolonized phloem and phloem colonized by *G. clavigera* only or *O. montium* only (Fig. 4); only insects from DeBorgia demonstrated a preference for phloem colonized by one

of the fungal associates over un-colonized phloem. The lack of choice displayed by the Butte larvae is surprising if phloem colonized by hyphae is indeed more nutritious because foraging theory predicts that insects will preferentially feed on higher quality diets (MacArthur and Pianka 1966). However, other studies have found that while insects usually choose to feed on higher quality diets in laboratory experiments and in the field, they do not always select the most nutritious food source as factors other than nutrient content may also influence feeding preference (e.g., Kursar et al. 2006, Scheu and Simmerling 2004). The isolates of *G. clavigera* and *O. montium* used in the Butte trial were darker and apparently possessed more melanin than the isolate of *G. clavigera* and *O. montium* used in the DeBorgia trial, which could affect insect feeding preference (Scheu and Simmerling 2004). Regardless, larvae from both populations displayed a general preference for phloem colonized by *G. clavigera* and *O. montium* together over un-colonized phloem or phloem colonized by one fungus (with one exception where there was no preference) (Fig. 4). This suggests that the fungal associates may provide complementary versus redundant benefits. These benefits may be in addition to: (1) increased size, because in the field study beetles emerging carrying *G. clavigera* only were just as large as beetles carrying both fungi together; and (2) increased nitrogen, because nitrogen levels did not differ among treatment diets in phloem sandwiches. Additional or complementary benefits provided by the two fungi growing together may include detoxifying phloem chemistry (Paine et al. 1997) or providing factors critical for developing insects, such B-group vitamins and ergosterol (Beaver 1989, Bentz and Six 2006).

Conidia lining the pupal chamber were consumed by MPB adults during pre-emergence feeding in the experiment where larvae and teneral adults were switched between *O. montium* and un-colonized phloem diets, regardless of larval diet. When spores were not present to feed on, MPB teneral adults usually compensated by mining extensively in the phloem before emerging. MPB teneral adults have been observed to feed on conidia lining pupal chambers previously and this feeding was suggested to be necessary to support beetle reproduction (Whitney 1971, Six and Paine 1998). Six and Paine (1998) observed that MPB teneral adults that did not feed on conidia were much less likely to produce egg galleries and none laid eggs. However, despite the potential nutritional benefits, pre-emergence feeding on the spores of ophiostomatoid fungi is apparently not a prerequisite for reproduction because three pairs of MPB adults that were reared from eggs in un-colonized PSs were introduced into short logs and they produced brood adults (K. Bleiker, unpublished data). Teneral adults of *I. pini* and *I. grandicollis* have also been observed feeding on fungi sporulating in pupal chambers, although their potential dietary importance was not recognized at the time (Leach et al. 1934). The nutrients gained during the pre-emergence feeding period may be essential for exoskeleton hardening, reproductive maturation, wing muscle development, fat storage, hormone production, and pheromone biosynthesis (McNee et al. 2000 and references therein).

Implications of nutritional fungal symbionts to bark beetles. Based on the results of this study, the fungal associates of the MPB may have significant impacts on MPB population dynamics. Bark beetle size is positively correlated with survival (Safranyik 1976), dispersal ability (Atkins 1967), fat content (Atkins 1967) and fecundity

(McGhehey 1971). In the DeBorgia population where a significant number of beetles emerged without ophiostomatoid fungi, the size difference between female beetles without ophiostomatoid fungi and those carrying *G. clavigera* only (1.80 and 2.05 mm, respectively) could translate into a 300% difference in the number of eggs laid (38 vs. 115 eggs per female) (McGhehey 1971). The size difference between females emerging with *G. clavigera* only and *O. montium* only could translate into a 28% (115 vs. 90 eggs per female) and 10% (115 vs. 105 eggs per female) difference in the number of eggs laid for Butte and DeBorgia populations, respectively. Thus, the presence and relative abundances of *G. clavigera* and *O. montium* in a beetle population could influence the rate of population growth. The relative abundances of the fungi in a population, and factors that affect the abundances of the fungi, could be especially important in determining how quickly endemic MPB populations respond to favorable conditions (e.g., favorable weather, after a stress event lowers tree resistance) and whether they erupt into outbreaks. In fact, it has been hypothesized that population trends of *D. frontalis* may be related to the abundances of its mutualistic mycangial fungi (Bridges 1985, but see Hofstetter et al. 2006a). Population trends of *D. frontalis* have been related to the abundance of an antagonistic, non-mycangial, ophiostomatoid fungus that may be carried on the beetle's exoskeleton and by phoretic mites associated with the beetle (Bridges 1985; Lombardero et al. 2003; Hofstetter et al. 2006a, 2006b).

Biotic and abiotic factors may interact and potentially change the strength or even the nature of interspecies interactions (Thompson 1997). Other species in the system, e.g., yeasts or bacteria, as well as environmental conditions, such as temperature, or phloem nutrient or moisture content that affect the fungi, may affect the direction or strength of

bark beetle-fungal interactions. This conditionality or context-dependency has been recognized in interspecies interactions (e.g., Bronstein 1994), including in bark beetle-fungal associations (Klepzig and Six 2004). Based on the field study, the two fungi appear to be mutualists of the MPB and offer redundant benefits in terms of increased size; however, the magnitude of the benefit differs. Thus, *O. montium* could be considered an antagonist of the MPB if it competes with *G. clavigera* and reduces interactions between MPBs and *G. clavigera*. However, based on the preference of the MPB for phloem colonized by both fungi together in the choice assay, *G. clavigera* and *O. montium* may offer the beetle complimentary benefits, at least under certain conditions. The MPB may also benefit from multiple associates as the different ecological niches of the fungi (e.g., temperature or moisture tolerances) may reduce the possibility the beetle is left without a symbiont if conditions are unfavorable for one of its partners (Six and Bentz 2007, Hofstetter et al. 2007). Ecological differences among mutualists may promote stability for the host in a variable world and allow multiple mutualists to coexist by reducing competitive interactions among similar species (Stachowicz and Whitlatch 2005).

Craighead (1928) first proposed that the relationship between bark beetles and fungi was mutualistic. He outlined a potential role of fungi in tree death and conditioning the host tree for brood development and he also suggested that the fungi may provide nutritional benefits to the beetles. However, since 1928, the overwhelming emphasis on bark beetle-fungal interactions has been on the first role Craighead proposed, likely due to the economic importance of tree mortality agents. The results of this study provide evidence that fungal associates may supplement the diet of bark beetles, which may allow

them to develop in a nutritionally-poor habitat at high densities. Development time is apparently more heavily influenced by factors other than diet quality, such as temperature (Bentz et al. 1991). The effect of diet quality on phloem consumption varied in the larval life stage, but teneral adults without conidia to feed on compensated by consuming more phloem. Both exogenous factors, such as climate, and endogenous factors, such as beetle numbers, tree resistance, intra- or inter-specific competition, and interactions among fungal associates, could result in variation in the benefits provided by multiple associates. While interactions with host trees and their defenses have undoubtedly had a major influence on the evolution of bark beetles, nutritional symbioses with ophiostomatoid fungi are also likely to have played a major role (Farrell et al. 2001).

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Table 1. Effect of fungal diet on spore production in pupal chambers during eclosion of adult mountain pine beetles reared in phloem sandwiches.

Diet ¹	2004 Trial			2005 Trial		
	Beetles (<i>n</i>)	No Conidia (%)	Conidia Present (%)	Beetles (<i>n</i>)	No Conidia (%)	Conidia Present (%)
C	10	50	50	29	100	0
M	20	5	95	11	91	9
CM	13	8	92	--	--	--

¹ C = *G. clavigera*-colonized phloem; M = *O. montium*-colonized phloem; CM = *G. clavigera*- and *O. montium*-colonized phloem.

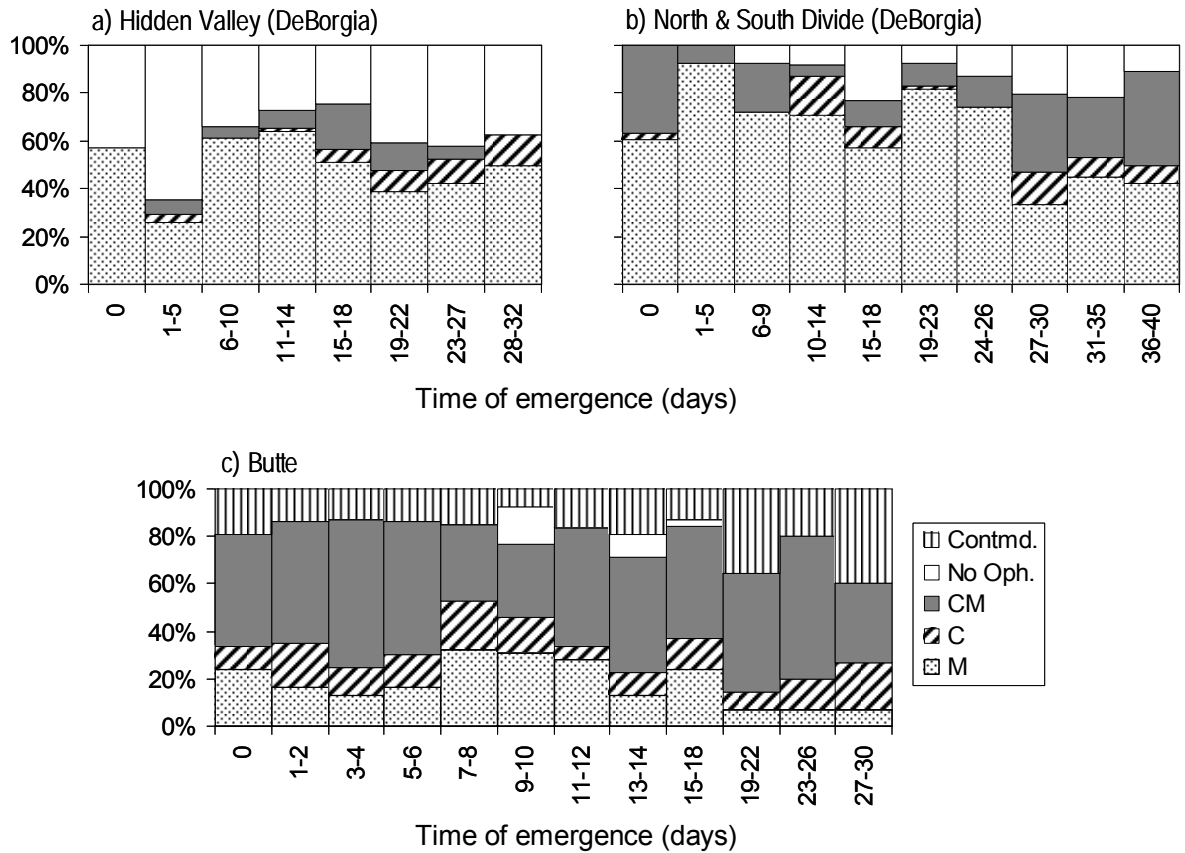


Fig. 1. Percent of mountain pine beetles emerging with different fungal complements from (a) Hidden Valley (DeBorgia), (b) North and South Divide sites (DeBorgia) and (c) Butte in western Montana. Collections were combined into 4 or 5 day periods for the DeBorgia sites. Collections were combined into 2 d periods for the Butte population for the first two weeks of the emergence period and then into 4 d periods for the last two weeks because of low numbers of beetles emerging. In the DeBorgia population, sampling was arrested due to wildfires before the emergence period at the North and South Divide sites was complete so these data are presented separately from the Hidden Valley site. Fungal complements are based on the sum of the fungi isolated from the two mycangia and are coded as follows: C = *G. clavigera* only; M = *O. montium* only; CM = both *G. clavigera* and *O. montium*; No Oph. = no ophiostomatoid fungi; and Contmd. = contaminated (ophiostomatoid species obscured). DeBorgia beetles were collected in emergence traps in the field; Butte beetles were collected from logs held indoors. Sample sizes (*n*) are as follows: Hidden Valley, 369 beetles; South and North Divide, 600 beetles; and Butte, 335 beetles.

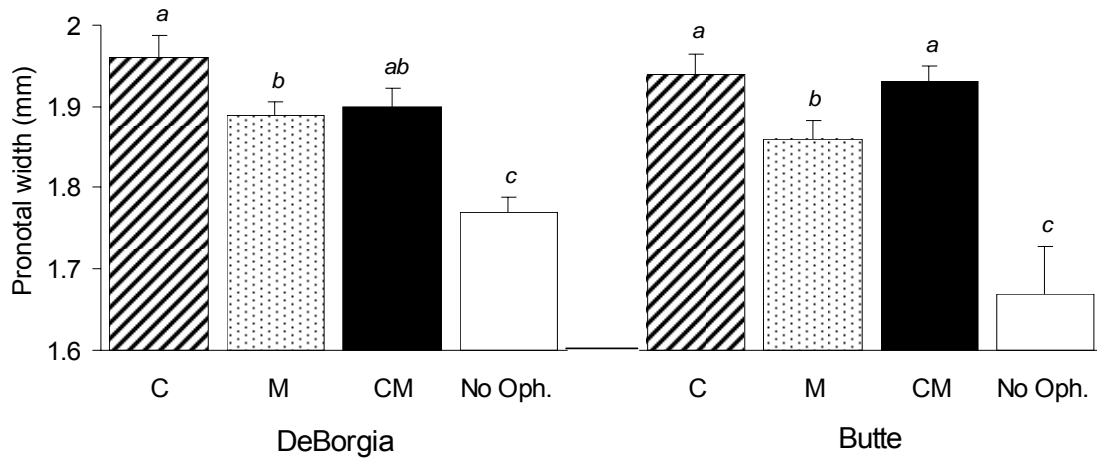


Fig. 2. Pronotal width of mountain pine beetles emerging carrying different fungal complements from DeBorgia and Butte populations. Fungal complements are coded as follows: C = *G. clavigera* only; M = *O. montium* only; CM = both *G. clavigera* and *O. montium*; and No *Oph.* = no ophiostomatoid fungi. LS means +SE are shown. Fungal complements with the same letter within a population are not significantly different at $P \leq 0.05$. Sample sizes for fungal complements in the order they appear on the x-axis are: DeBorgia: 40, 311, 68, and 166; and Butte: 45, 55, 125, and 6.

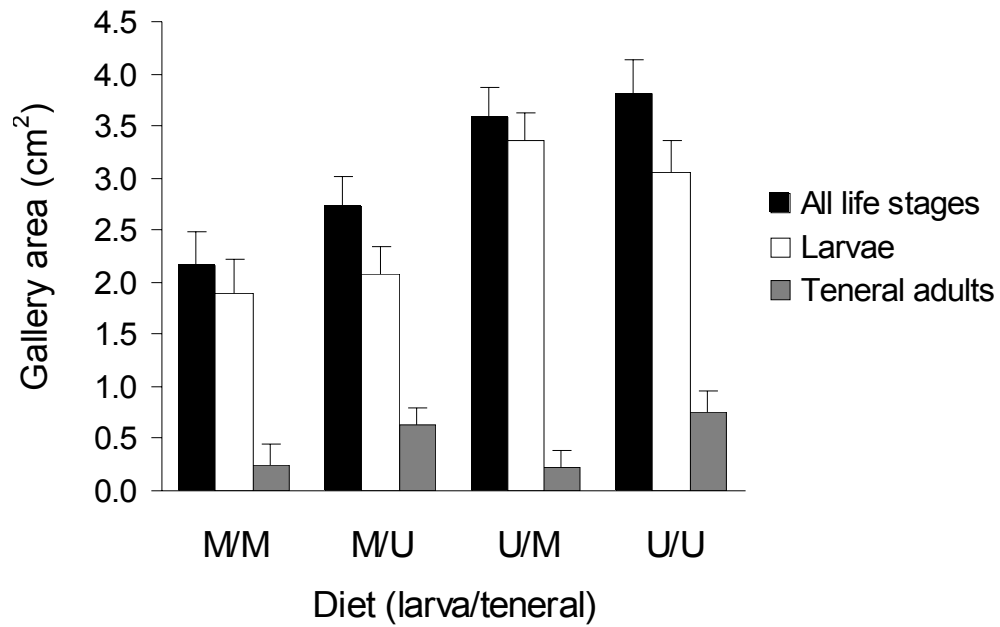


Fig. 3. Effect of larval and teneral adult diet on the amount of phloem consumed by mountain pine beetle life stages developing with and without *O. montium*. Newly eclosed adults were switched between treatment diets (M = *O. montium*-colonized phloem; and U = un-colonized phloem). All life stages includes larval and teneral adult life stages combined. LS means +SE are shown. Sample sizes (*n*) for each diet combination in the order presented on the x-axis are: 6, 9, 8, and 8.

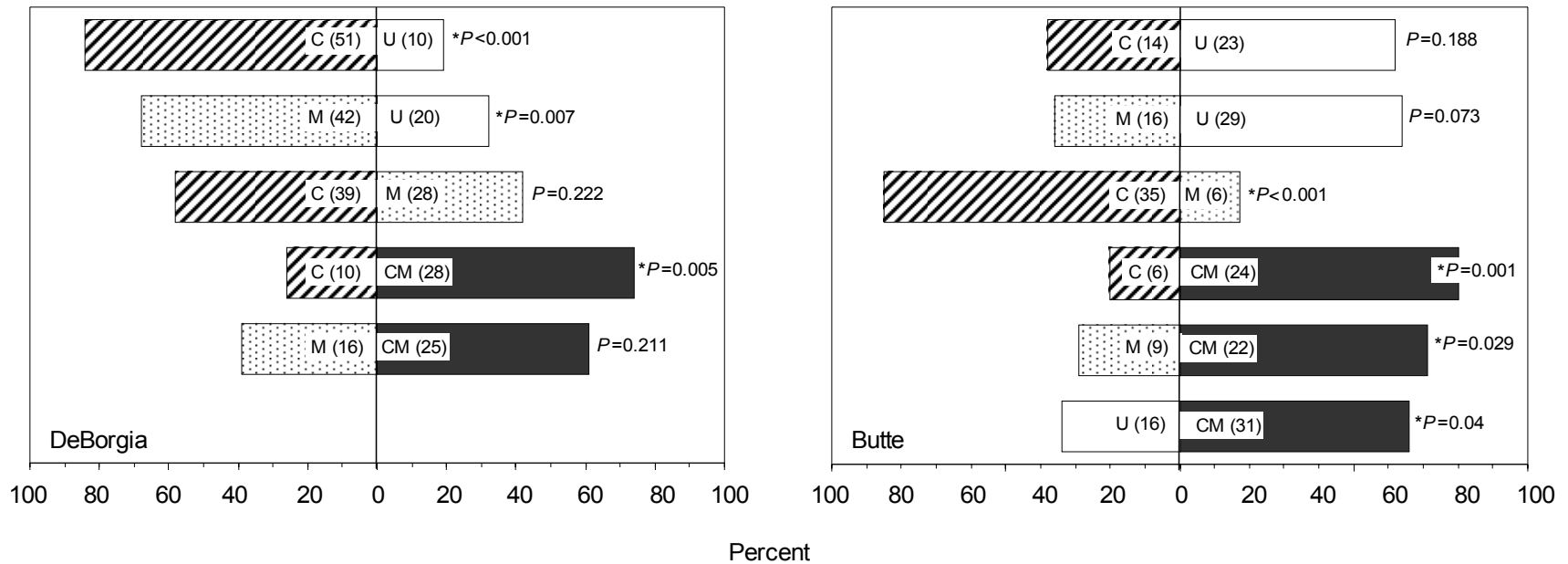


Fig. 4. Effects of the fungal associates of mountain pine beetle on the feeding preferences of larvae from two populations in western Montana. Sample sizes (n) are in brackets following the food choices, which are coded as follows: U = un-colonized phloem; C = *G. clavigera*-colonized phloem; M = *O. montium*-colonized phloem; and CM = *G. clavigera*- and *O. montium*-colonized phloem. * Denotes significant binomial probability at $P \leq 0.05$.

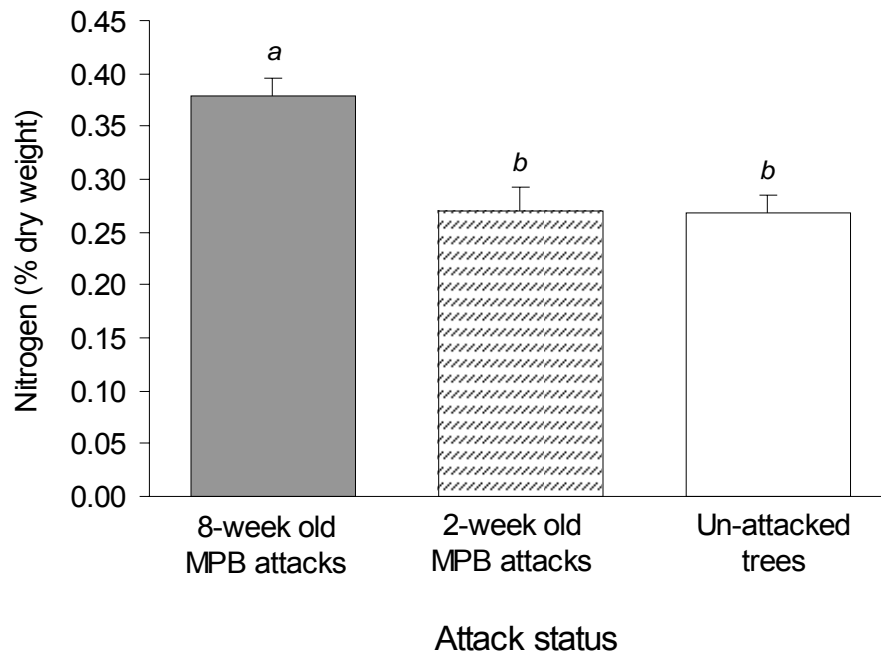


Fig. 5. Nitrogen content of phloem from lodgepole pine trees attacked by mountain pine beetle approximately eight and two weeks prior to sampling as well as un-attacked trees. Means +SE are shown and attack status classes with the same letter are not significantly different at $P \leq 0.05$. The number of trees sampled for each attack status class in the order listed on the x-axis were 15, 9, and 15.

Chapter 2

Effects of water potential and solute on the growth and interactions of two fungal symbionts of the mountain pine beetle

Abstract

I investigated the effect of water potential (WP) on the growth of, and interaction between, two ophiostomatoid fungi, *Grosmannia clavigera* and *Ophiostoma montium*, associated with the mountain pine beetle (*Dendroctonus ponderosae*). The WP of malt extract agar was amended by adding KCl or sucrose. Growth of both fungi decreased with WP on KCl-amended media. Growth of *G. clavigera* also decreased with WP on sucrose-amended media, although growth was stimulated on these media compared to un-amended treatments. Growth of *O. montium* remained relatively constant on sucrose-amended media, confounding the effect of WP on this species. Both fungi were able to colonize media occupied by the other species, but at a slower rate than on un-occupied media, indicating competition. In most treatments, *G. clavigera* grew faster than *O. montium* and colonized a greater area when the two fungi were inoculated concurrently but distant to one another on a Petri dish. However, when each fungus was inoculated adjacent to a 10 day old well-established colony of the other species, *O. montium* colonized occupied media more effectively than *G. clavigera* considering the growth rate of each species alone. Thus, *G. clavigera* dominated primary (un-colonized) resources on most media treatments, but *O. montium* was more effective in colonizing secondary (occupied) resources. The differential response of the two fungi to sucrose indicates that they may use different carbon sources, or use different carbon sources at different rates, in the tree. Fine scale resource partitioning, differences in primary and secondary resource capture abilities, and the non-equilibrium dynamics in an attacked tree over time, could all act to promote the co-existence of two unit-restricted dispersers on a discontinuous resource.

Introduction

The importance of competition in shaping ecological communities is well known. Competition occurs when one species negatively affects another by consuming a common limited resource (exploitation) or controlling access to a limited resource (interference) (Lockwood 1981; Wicklow 1981). These types of competition are difficult to dissociate for many fungi because nutrient acquisition is often dependent upon competition for space for fungi (e.g., Boddy 2000). As a result, competition between fungi has been categorized as either primary resource capture (colonization of unoccupied habitat) or secondary resource capture (colonization of habitat that is already occupied) (Rayner & Webber 1984). Regardless of the terms applied, most research on competitive interactions has focused on plants growing at high densities and on animals competing for the same resource; comparatively less is known about the effect of competition on fungal communities (Shearer 1995).

Many fungi in the Ophiostomataceae (Ascomycotina) have symbiotic associations with phloeophagous bark beetles (Coleoptera: Curculionidae, Scolytinae). Often more than one species of fungus is associated with a single bark beetle species (reviewed in Six 2003a). The fungi are dependent on the beetle for transport to new trees that are either recently dead or moribund after being attacked *en masse* by beetles. Fungal spores are carried by adults on the exoskeleton or in mycangia (structures of the integument for transporting microorganisms) (Beaver 1989) and inoculated into tree tissues during construction of egg galleries in the phloem and inner bark. The fungi rapidly colonize the phloem and sapwood of trees successfully attacked by beetles (e.g., Reid

1967; Solheim 1995; Chapter 3). Teneral (young, sexually immature) adults acquire fungal spores just prior to dispersing from the natal host when they pre-emergence feed on thick layers of spores that commonly line the pupal chambers (Whitney 1971; Six & Paine 1998; Bleiker & Six accepted). Thus, sporulation in pupal chambers must coincide with the beetle's pre-emergence feeding in order for the fungi to be transported to the next host tree. Conditions change rapidly in trees following attack, which may affect sporulation (Mathiesen-Käärrik 1960). Resources (e.g., nutrients and moisture) may also be limited by the time beetles are pre-emergence feeding (Klepzig *et al.* 2004; Kim *et al.* 2005; Chapter 3) potentially affecting the ability of the fungi to sporulate in the pupal chambers. Limited resources in the tree and variation in resource use or requirements of different species of fungi (Mathiesen- Käärrik 1960) may affect the ability of fungi to compete for resources and sporulate in the pupal chambers.

In beetle-attacked trees, the ophiostomatoid fungal associates apparently share the same limiting resources, typically easily assimilable nutrients (Seifert 1993). Thus, the fungi may be expected to compete with one another when growing together. Competition may result in species persisting on a resource at some equilibrium level, or in competitive exclusion where the victor is the species that can survive on the lowest level of a resource (e.g., Gause 1934). These classic outcomes of competition may be most applicable to systems where resources are, at least periodically (e.g., seasonally), replenished. However, a beetle-attacked tree is a discontinuous resource (or unit) for the fungi: resources within the tree decline over time, which results in non-equilibrium conditions (Schmit 1999). Once resources within a tree are consumed, the individual fungi that inhabit the tree will die because they inhabit a discrete resource (Rayner & Webber

1984). Ophiostomatoid fungi do not disperse vegetatively (e.g., using rhizomorphs or mycelium to locate new habitat patches); instead, they are unit-restricted dispersers (Rayner 1994) that rely on bark beetles for transport to the next suitable habitat. Thus, the challenge for the fungi is twofold: (1) capture sufficient phloem resources to maximize contact with insects in pupal chambers; and (2) coordinate sporulation in the pupal chambers with the beetle's pre-emergence feeding period, a time when resources (e.g., moisture) are most likely to be limited.

In this study, I examine interactions between two ophiostomatoid fungi, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield (previously *Ophiostoma clavigerum*) and *Ophiostoma montium* (Rumbold) von Arx, which are commonly associated with the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (e.g., Whitney & Farris 1970; Whitney 1971; Six 2003b; Bleiker & Six accepted). The mountain pine beetle is an eruptive, tree-killing bark beetle that causes significant economic losses in mature pine forests in western North America (Safranyik & Carroll 2006). The fungi may aid the beetle in overwhelming tree defenses and conditioning phloem (e.g., detoxifying tree defense chemicals, altering water relations) for brood development (Reid *et al.* 1967; Ballard *et al.* 1982, 1984; Raffa and Berryman 1983; Owen *et al.* 1987; Solheim 1995). *Grosmannia clavigera* is described as moderately pathogenic and can tolerate lower oxygen conditions compared to *O. montium*, and may be the primary invader of tree tissues (Solheim 1995; Solheim & Krokene 1998). *Grosmannia clavigera* also grows faster than *O. montium* at temperatures between 3 and 22°C; however, *O. montium* grows faster than *G. clavigera* at temperatures above 27 °C (Six & Paine 1997; Solheim & Krokene 1998). Both species appear to

benefit the beetle through nutritional supplementation; however, *G. clavigera* confers greater benefits than *O. montium*. More brood were produced by insects developing with *G. clavigera* compared to *O. montium* (Six & Paine 1998), and *G. clavigera*-carrying beetles emerging from attacked trees were larger than beetles carrying *O. montium* (Bleiker & Six in press). Given the difference in the magnitude of the benefits provided by the two fungi to the beetle, competitive interactions between the fungi could indirectly affect the beetle's fitness by altering the degree of contact beetles have with each species.

The objectives of this study were two-fold: (1) determine the nature of the interaction between *G. clavigera* and *O. montium*; and (2) determine the effect of water potential (WP) on the growth of each fungus and on interspecific fungal interactions. I tested the nature of the interaction between *G. clavigera* and *O. montium* by growing each fungus alone and with each other under controlled conditions in the laboratory. Because one of the most prominent changes in beetle-attacked trees over time is a decrease in moisture content, I tested for an effect of WP on the ability of each fungus to colonize un-occupied resources (primary resource capture) and resources already occupied by the other species (secondary resource capture) (Rayner & Webber 1984).

Materials & Methods

Fungal Cultures

Cultures of *G. clavigera* and *O. montium* used in the following experiments were isolated during a previous study (Bleiker & Six accepted) from the maxillae (includes the mycangia) of mountain pine beetles emerging from beetle-attacked lodgepole pine (*Pinus contorta* var. *latifolia* Dougl. Ex Loud) trees. A representative culture, in terms of

morphology and growth rate on 2% malt extract agar (MEA), of each species was selected. Inocula used in the following experiments were 4 mm diameter plugs taken from the actively growing margins of cultures maintained on MEA. All cultures and experiments were maintained at 21°C, except where noted.

WP treatments

Three media types were used: un-amended MEA, KCl (potassium chloride)-amended MEA, and sucrose-amended MEA. Each amended medium included six target WPs: 0, -0.25, -0.5, -1.0, -2.0, -3.0 MPa (available water declines with decreasing WP). Amending media with solutes and estimating the WP of the media using the theoretical osmotic potentials of the solutes and MEA has been used extensively in studies examining the effect of available water on fungal growth (e.g., Boddy 1983; Whiting & Rizzo 1999; Hong & Michailides 1999; Whiting *et al.* 2001; Klepzig *et al.* 2004). Two different solutes were used (separately) to ensure that the any response by the fungi was due to an effect of WP and not from stimulation or inhibition by the solute (Griffin 1977). The media were allowed to equilibrate for three days before fungal treatments were added on day 0 of the experiment. A WP4 Dewpoint PotentialMeter (Decagon Devices, Inc., Pullman, WA) was used to measure the actual WP of uninoculated media in three Petri dishes from each treatment at day 0 to assess the accuracy of the methods in achieving the target WPs. Because actual WP varied from the target WP (see Results, Table 1), I hereafter refer to treatments by their actual WPs. To determine if WP of the media changed significantly over the duration of the experiments, I also measured the actual WP of media in three Petri dishes from three KCl treatments and two sucrose treatments at day 30.

I used *t*-tests to test for changes in WP of media between days 0 and 30. These analyses, as well as all others listed below, were conducted using JMP[®] 5.1 (SAS Institute, Cary, NC, USA). Significance was declared at $P \leq 0.05$ for all tests.

Effect of media type and WP on fungal growth

To determine the effect of solute and WP on fungal growth, I inoculated the center of media in six 15 cm diameter Petri dishes from each of the 13 treatments with each fungus alone. Petri dishes were sealed with parafilm and held at 21°C. After 9 d, the diameter of each fungal colony was measured along two perpendicular axes. The first axis was randomly selected and the average of the two measurements was recorded for each Petri dish.

I used a *t*-test to test for a difference in growth of *G. clavigera* and *O. montium* on un-amended MEA. Variation in fungal growth on KCl- and sucrose-amended media was assessed using two-factor ANOVAs. Because the actual WP of the two amended media types were not identical (see Results, Table 1), the effect of WP on fungal growth was analyzed separately for each media type. Fungal species, actual WP, and the interaction between fungal species and actual WP were included as factors in the model. These analyses, as well as other ANOVAs and MANOVAs listed below, were conducted using a General Linear Model (GLM) approach. Significant *F* tests were followed by Tukey-Kramer's Honestly Significant Difference (HSD) test.

Effect of media type and WP on fungal interactions

To determine the effect of WP on interactions between *G. clavigera* and *O. montium*, I conducted two experiments in which the two fungi were inoculated onto media together. In one experiment, the fungi were inoculated concurrently but distant to

one another on media in Petri dishes. In this experiment, the fungi colonized un-occupied media (primary resource capture) until the leading margins of the colonies encountered each other after which time the fungi engaged in secondary resource capture. In a second experiment, inoculations were staggered in time so that one species was added to media already fully colonized by the other species (secondary resource capture).

Concurrent, distant inoculations

Grosmannia clavigera and *O. montium* were inoculated opposite each other (C--M), approximately 12 cm apart, on 14 cm diameter Petri dishes. *Grosmannia clavigera* and *O. montium* were also inoculated alone in the same location near the edge of a Petri dish as a control (C-- and M--, respectively). Each of the three fungal treatments was replicated six times on each of the 13 WP treatments.

The outline of each fungal colony was traced onto clear plastic sheets at three day intervals, starting on day 3 and ending on day 21. It was usually possible to visually locate the boundary of each fungal colony as it advanced into media already colonized by the other species because of a small change in the darkness of the media (lighter or darker). However, eventually two to three bands varying in darkness appeared on some treatments. In such cases, a compound microscope was used to examine samples from the different zones of darkness for spores and hyphae (Upadyay 1981). The boundary of *G. clavigera* was easily determined using this approach because it sporulates readily on MEA; however, *O. montium* does not. In addition, even though hyphae of the two species overlap in size, cultures of *G. clavigera* typically include some large, straight hyphae, which are easily distinguishable from the smaller, more kinked hyphae of *O. montium*. Thus, it is possible to identify some *G. clavigera* hyphae amongst *O. montium* hyphae,

but it is more difficult to identify *O. montium* hyphae amongst *G. clavigera* hyphae given the wider size range of *G. clavigera* hyphae (Upadyay 1981). Thus, to verify the boundary of *O. montium*, all zones were traced onto clear plastic sheets and two 2 mm squares of media spanning each zone were placed onto media (MEA) in separate Petri dishes. One set of dishes was incubated at 29°C and the other at 19°C for four days. The isolates of *G. clavigera* and *O. montium* used in this study had noticeably different growth rates at these temperatures, which are characteristic of these two fungi (Six & Paine 1997; Solheim & Krokene 1998). The isolate of *Ophiostoma montium* used in this study grew rapidly at 29°C, whereas growth of the *G. clavigera* isolate was negligible at this temperature after 4 d. While both fungi grew well at 19°C, *G. clavigera* grew noticeably faster than *O. montium*. Thus, it was possible to determine which zones were colonized by *O. montium* and determine the correct boundary, as well as corroborate the boundary of *G. clavigera* by assessing fungal growth at the two temperatures after four days.

The clear plastic sheets with tracings of the fungal colony boundaries were photographed with a digital camera secured at a constant height above the sheets. I used Adobe® Photoshop® v. 9 (Adobe Systems, Inc., San Jose, California, USA) to calculate the number of pixels in the areas colonized by *G. clavigera* only, *O. montium* only, and both fungi together for each of the 234 Petri dishes at each of the 7 sample times. The number of pixels in each area was converted to cm² by dividing by the number of pixels in 1 cm².

A repeated measures MANOVA was used to test for the effects of fungal treatment (i.e., growing alone and with the other species), and actual WP on the growth

of each fungal species over time. All interactions, including those with time, were included in the analysis. Approximate *F* values are reported for MANOVAs and significant results were followed by univariate ANOVAs to determine which factors contributed to the difference. Significant ANOVAs were followed by Tukey-Kramer's HSD test for all factors except actual WP. The effect of actual WP on the growth of *G. clavigera* and *O. montium* was consistent with the results of the previous experiment (effect of WP on radial growth of fungi), and thus, to avoid redundant results, *post hoc* analysis was not conducted on this factor.

Staggered, overlapping inoculations.

To determine the ability of *Grosmannia clavigera* and *O. montium* to colonize media already occupied by the other species, I inoculated each fungus alone into the center of media in 9 cm diameter Petri dishes and held the cultures at 21°C. After 10 days, the other species was inoculated in the center of the dish immediately adjacent to the first fungus, resulting in two fungal treatments: *G. clavigera* with *O. montium* added at day 10, C(+M); and *O. montium* with *G. clavigera* added at day 10, M(+C). Each fungal treatment was replicated 5 times on each WP treatment.

As controls, *G. clavigera* and *O. montium* were also inoculated alone into the center of media in 5 Petri dishes of each WP treatment at day 10. The number of days it took each species to fully colonize all 5 Petri dishes from the same WP treatment was recorded as 'X' days and the corresponding WP treatment that had that species added on day 10 was sampled (sampling method described below) (Table 2). Treatments were also sampled a second time, which was an additional 'X' days after the first sample time (Table 1).

At each sample time, two 2 mm squares of media were removed from six sample locations along an axis that bisected both inoculation plugs in the center of the Petri dish. The sample locations mirrored each other along the axis on each side of the two plugs and were as follows: adjacent to each inoculum plug, mid-way between each plug and the Petri dish edge, and adjacent to the Petri dish edge. The paired samples were placed on MEA in separate Petri dishes and one set was incubated at 29°C and the other held at 19°C. After four days the Petri dishes were examined and the presence/absence of each fungal species at each location determined using the methods described above.

The ability of *G. clavigera* and *O. montium* to colonize media already occupied by the other species was assessed using a visual display of the frequency with which each fungus was isolated from the different sample locations from the 5 replicates for each treatment. No statistical analysis was performed on these data because multiple cells contained less than five observations violating the assumptions of frequency analysis.

Results

WP of media

The mean actual WP of un-amended MEA was -0.13 MPa. The actual WP of media amended with KCl or sucrose measured with the Dewpoint PotentialMeter differed from the theoretical (target) WP by 0.24 to 0.80 MPa depending on the treatment (Table 1). The WP of both types of amended media tended to be lower than the theoretical WP. Hereafter, I use the actual WP of the treatments reported in Table 1.

The mean WP of un-amended MEA did not change between day 0 and day 30 ($t_5=0.60$, $P=0.58$). The WP of most of the amended media treatments that were measured

on both day 0 and 30 also did not change over time (KCl -1.4 MPa: $t_5=-2.27$, $P=0.07$; KCl -3.3 MPa: $t_5=0.83$, $P=0.44$; sucrose -2.7 MPa: $t_5=-1.52$, $P=0.19$; and sucrose -3 MPa: $t_5=2.35$, $P=0.07$). Only the WP of KCl -0.2 MPa changed significantly after 30 days, increasing to -2.0 MPa ($t_5=-2.04$, $P\leq 0.05$); however, the small change (+0.23 MPa) was likely only significant because of the low variation and the small sample size (Table 1).

Effect of medium on fungal growth

The addition of small amounts of solutes (KCl or sucrose) to MEA increased fungal growth, but high amounts of these same solutes had a negative affect on fungal growth (Figure 1). Growth of both *G. clavigera* and *O. montium* was greater on sucrose-amended media than on KCl-amended media at a given WP; however, the magnitude of the difference varied by species and WP (Figure 1). Growth of *G. clavigera* was consistently two to three times higher on sucrose-amended media than on KCl-amended media at the same WP (Figure 1). While growth of *O. montium* was also higher on sucrose-amended media than on KCl-amended media at a given WP, the difference was greatest at the lower WPs. This was due to *O. montium* growth remaining relatively constant across most WPs on sucrose-amended media, while declining with WP on KCl-amended media (Figure 1).

Effect of WP on fungal growth

Un-amended media. On un-amended MEA (-0.13 MPa), growth of *G. clavigera* after 9 d was greater than growth of *O. montium* ($t_{10}=5.92$, $P<0.0001$) (Figure 2).

KCl-amended media. *Grosmannia clavigera* grew faster than *O. montium* on media amended with KCl at the same WP ($F_{1,58}=395.22$, $P<0.0001$) (Figure 2). WP affected fungal growth ($F_{4,58}=783.41$, $P<0.0001$). Growth of *G. clavigera* did not differ at

the two highest WPs (-0.4 and -0.6 MPa) ($P>0.05$), but declined with each successive WP below -0.6 MPa ($P\leq 0.05$) (Figure 1). Growth of *O. montium* declined with each successive drop in WP ($P\leq 0.05$). Overall, growth of both fungi declined in a similar manner as WP decreased (Figure 1); however, the WP by fungal species interaction was significant ($F_{4,58}=7.46$, $P<0.0001$). The interaction was likely due to the curvilinear response of *G. clavigera*, compared to the linear response of *O. montium*, at WPs between -0.4, -0.6, and -0.8 MPa.

Sucrose-amended media. Fungal species, actual WP, and the fungal species by actual WP interaction affected fungal growth on media amended with sucrose ($F_{1,68}=529.08$; $F_{5,68}=345.53$; and $F_{5,68}=159.35$, respectively, $P<0.0001$ for all).

Grosmannia clavigera grew much faster than *O. montium* on sucrose-amended media at WPs of -0.8, -1.0, -1.1, and -1.5 MPa; however, *O. montium* grew faster than *G. clavigera* at -2.7 and -3.3 MPa ($P\leq 0.05$) (Figure 3). On media amended with sucrose, the two fungi responded differently to changes in WP. Growth of *G. clavigera* did not differ at the two highest WPs (-0.8 and -1.0 MPa), but growth of *G. clavigera* declined at each successive WP below -1.0 MPa ($P\leq 0.05$). In contrast, growth of *O. montium* varied little across the range of WPs, with the only differences being that *O. montium* grew slower on -3.3 MPa than on -0.8 and -2.6 MPa ($P\leq 0.05$) (Figure 3). These differences were actually quite small, but were significant because variation in fungal growth (within a species) among replications in a treatment was extremely low.

Effect of WP on fungal interactions

Concurrent, distant inoculations

Un-amended media. Overall, growth of *G. clavigera* was not affected by the presence of *O. montium* on un-amended MEA ($F_{1,10}<0.1$, $P=0.94$) (Figure 2a); however, the fungal treatment (C--M, C--, M--) by time interaction term was significant ($F_{6,5}=31.0$, $P=0.0008$). At day 9 more media was colonized by *G. clavigera* when it was growing with *O. montium* ($F_{1,10}=8.13$, $P=0.02$), but at days 18 and 21, more media was colonized by *G. clavigera* when it was growing alone ($F_{1,10}=9.8$, $P=0.01$; $F_{1,10}=50.1$, $P<0.0001$) (Figure 2a). There were no differences between the two treatments in the area colonized by *G. clavigera* at days 3, 6, or 15 ($F_{1,10}=4.3$; $F_{1,10}=3.9$; and $F_{1,10}=1.3$, respectively, $P>0.05$ for all). Time had an effect on the area colonized by fungus as the colonies grew over time ($F_{6,5}=40047.9$, $P<0.0001$).

Fungal treatment affected growth of *O. montium* ($F_{1,10}=89.0$, $P<0.0001$) although the effect varied over time ($F_{6,5}=333.4$, $P<0.0001$) (Figure 2a). The area colonized by *O. montium* did not differ between the two fungal treatments at days 3, 6, and 9 when un-occupied medium in the dishes was still being colonized ($F_{1,10}=0.6$; $F_{1,10}<0.1$; and $F_{1,10}=0.3$, respectively, $P>0.05$ for all). However, at days 12, 15, 18, and 21, when *O. montium* encountered medium already colonized by *G. clavigera*, it colonized less area than when it was growing alone ($F_{1,10}=147.4$; $F_{1,10}=523.7$; $F_{1,10}=8.1$; and $F_{1,10}=1557.8$, respectively, $P<0.02$ for all) (Figure 2a). The area colonized by fungus in all treatments increased over time ($F_{6,5}=3670.8$, $P<0.0001$).

KCl-amended media. Overall, growth of *G. clavigera* was not affected by fungal treatment ($F_{1,36}<0.1$, $P=0.99$). However, time and all interaction terms had an effect on the area colonized by *G. clavigera* (time: $F_{6,31}=13400.2$; time by fungal treatment: $F_{6,31}=65.5$; time by actual WP: $F_{18,88}=58.6$; fungal treatment by actual WP: $F_{3,36}=11.9$;

and time by fungal treatment by actual WP: $F_{18,88}=15.0$, $P<0.0001$ for all). At days 3 and 12, fungal treatment had no effect on the area colonized by *G. clavigera* in the two treatments ($F_{1,36}=1.4$, $P=0.23$; $F_{1,36}=0.7$, $P=0.51$, respectively). Fungal treatment did account for significant variation in the area colonized by *G. clavigera* at days 6, 9, 15, 18, and 21 ($F_{1,36}=40.0$; $F_{1,36}=58.4$; $F_{1,36}=61.0$; $F_{1,36}=47.8$; and $F_{1,36}=8.9$, respectively, $P\leq 0.005$ for all). However, the fungal treatment by actual water WP interaction was significant at each sample time from day 3 to 21 ($F_{3,36}=7.1$; $F_{3,36}=6.5$; $F_{3,36}=6.2$; $F_{3,36}=10.7$; $F_{3,36}=50.6$; $F_{3,36}=47.8$; and $F_{3,36}=12.9$, respectively, $P\leq 0.002$ for all). Initially, the area colonized by *G. clavigera* was greater in the treatment where it was growing with *O. montium*. However, once *G. clavigera* encountered media already colonized by *O. montium*, its growth slowed, and the area colonized by *G. clavigera* was greater in the treatment where it was growing alone. Although the day on which the two fungi encountered each other varied among WP treatments, the pattern of *G. clavigera* growing slightly faster with *O. montium* initially, but slowing when media already colonized by *O. montium* was encountered, was evident in all WP treatments (Figure 2), except -1.4 KCl. Fungal growth was so slow at KCl -1.4 MPa that *G. clavigera* and *O. montium* had only just encountered each other on the media at day 21.

Overall, growth of *O. montium* was affected by fungal treatment ($F_{1,37}=213.5$, $P<0.0001$). Time and all interactions also had an affect on the area colonized by *O. montium* in the two treatments (time: $F_{6,32}=7423.9$; time by fungal treatment: $F_{6,32}=113.7$; time by actual WP: $F_{18,91}=71.4$; fungal treatment by actual WP: $F_{3,37}=36.8$; and time by fungal treatment by actual WP: $F_{18,91}=12.5$, $P<0.0001$ for all). At days 3, 6, and 9, neither fungal treatment nor the fungal treatment by actual WP interaction had an affect on the

area colonized by *O. montium* (fungal treatment: $F_{1,37}=0.2$; $F_{1,37}=1.5$; and $F_{1,37}=0.14$; fungal treatment by actual WP: $F_{3,37}=1.0$; $F_{3,37}=1.4$; and $F_{3,37}=0.91$, respectively, $P>0.23$ for all). However, at days 12, 15, 18, and 21 both fungal treatment and the fungal treatment by actual WP interaction had an effect on the area colonized by *O. montium* (fungal treatment: $F_{1,37}=38.8$; $F_{1,37}=88.5$; $F_{1,37}=213.3$; and $F_{1,37}=436.7$; fungal treatment by actual WP: $F_{3,37}=12.0$; $F_{3,37}=16.9$; $F_{3,37}=34.1$; and $F_{3,37}=80.9$, respectively, $P<0.0001$ for all). *Ophiostoma montium* colonized the various media at a similar rate when it was growing with *G. clavigera* as it did growing alone until medium already colonized by *G. clavigera* was encountered. *Ophiostoma montium* continued to colonize medium already occupied by *G. clavigera* at a much slower rate than un-colonized medium. The trend of *O. montium* growth slowing once it encountered medium already occupied by *G. clavigera* was evident at KCl -0.4, -0.6, and -0.8 MPa, although the two fungi encountered each other on different days since fungal growth varied with WP (Figure 2).

Sucrose-amended media. The area colonized by *G. clavigera* was affected by fungal treatment ($F_{1,58}=21.3$, $P<0.0001$) (Figure 3); however, time and all interaction terms were significant (time: $F_{6,53}=1867.9$; time by fungal treatment: $F_{6,53}=41.7$; time by actual WP: $F_{30,214}=71.4$; fungal treatment by actual WP: $F_{5,58}=3.8$; and time by fungal treatment by actual WP: $F_{30,214}=10.6$, $P<0.005$ for all). At days 3, 6, 15, 18, and 21 and all WPs, fungal treatment had no effect on the area colonized by *G. clavigera* ($F_{1,58}=2.1$; $F_{1,58}=1.3$; $F_{1,58}=2.07$; $F_{1,58}=0.1$; $F_{1,58}=0.15$, respectively, $P>0.15$); fungal treatment only had an effect on area at day 9 ($F_{1,58}=23.9$, $P<0.0001$). There was no significant effect of the fungal treatment by actual WP interaction on the area colonized by *G. clavigera* at day 15 ($F_{5,58}=1.7$, $P=0.16$), but the interaction was significant at all sample times from

day 3 through 21 ($F_{5,58}=4.7$; $F_{5,58}=3.1$; $F_{5,58}=8.8$; $F_{5,58}=40.6$; and $F_{5,58}=6.8$, respectively, $P<0.01$ for all). The area colonized by *G. clavigera* when it was growing with *O. montium* was similar to when it was growing alone until *G. clavigera* encountered medium already colonized by *O. montium*. *Grosmannia clavigera* invaded medium colonized by *O. montium* at a slower rate than un-occupied medium. Thus, the area colonized by *G. clavigera* was greatest when it was growing alone at the later sample times (Figure 3). The trend of *G. clavigera* growth slowing once it encountered media occupied by *O. montium* was evident at all WPs, although the two fungi encountered each other on different days since fungal growth varied with WP.

The area colonized by *O. montium* was affected by fungal treatment ($F_{1,57}=356.1$, $P<0.0001$) (Figure 3); however, the effect of time and all interactions also had an effect (time: $F_{6,52}=991.3$; time by fungal treatment: $F_{6,52}=104.1$; time by actual WP: $F_{30,210}=27.0$; fungal treatment by actual WP: $F_{5,57}=52.8$; and time by fungal treatment by actual WP: $F_{30,210}=12.4$, $P<0.001$ for all). At days 3 and 6, the area colonized by *O. montium* did not vary with fungal treatment or the fungal treatment by actual WP interaction (day 3: $F_{1,57}=0.5$, $F_{5,57}=0.8$; and day 6: $F_{1,57}=1.9$, $F_{5,57}=1.5$, respectively, $P>0.2$ for all). However, fungal treatment and the fungal treatment by actual WP interaction had an effect on the area colonized by *O. montium* at days 9, 12, 15, 18, and 21 (fungal treatment: $F_{1,57}=36.9$; $F_{1,57}=178.0$; $F_{1,57}=290.2$; $F_{1,57}=402.1$; and $F_{1,57}=288.9$; fungal treatment by actual WP: $F_{5,57}=8.1$ $F_{5,57}=44.4$ $F_{5,57}=60.6$ $F_{5,57}=54.0$; $F_{5,57}=36.4$, respectively, $P<0.0001$ for all). Similar to its growth on KCl-amended media, the area colonized by *O. montium* did not differ between the two treatments until *O. montium* encountered medium already occupied by *G. clavigera*. *Ophiostoma montium* invaded

medium occupied by *G. clavigera* at a slower rate than un-colonized medium, and thus, *O. montium* colonized a greater area when it was growing alone at later sample times. Because fungal growth varied with WP, the day on which *O. montium* encountered media occupied by *G. clavigera* varied, resulting in interactions between fungal treatment and actual WP.

Staggered, overlapping inoculations.

After 10 days, growth of *G. clavigera* and *O. montium* on KCl -2.2 and -3.3 MPa was minimal and these treatments were dropped from the study. Both *G. clavigera* and *O. montium* were able to colonize media that was already occupied by a well-established colony of the other species (Figure 4). However, growth of both species was slower on medium colonized by the other species compared to when they were growing alone (Figure 4). Only *O. montium* growing on KCl -1.4 MPa was able to colonize medium already occupied by *G. clavigera* at the same rate as when it was growing alone (Figure 4). *Grosmannia clavigera* grew faster than *O. montium* when growing alone on all treatments except sucrose -2.7 and -3.3 MPa. Thus, sample times were longer for most C(+M) treatments (except sucrose -2.7 and -3.3 MPa) compared to M(+C) treatments (Table 2). Relative to growth rates on un-colonized media, *O. montium* was able to colonize media already occupied by *G. clavigera* faster than *G. clavigera* was able to colonize media already occupied by *O. montium* (Figure 4). By the second sample time, *O. montium* was isolated from all 6 sample locations on all 5 Petri dishes from every C(+M) treatment except sucrose -1.5 MPa (Figure 4). In comparison, by the second sample time, *G. clavigera* was isolated from fewer than 6 sample locations from the 5 dishes in each of the M(+C) treatments (Figure 4).

Discussion

Sucrose and KCl are commonly used in mycological studies, either alone or in combination, to study the effects of WP on fungal growth. This method assumes that the solutes have little effect on fungal growth independent of their effect on osmotic potential, especially when they are used in combination without controls for each solute. In order to eliminate potential effects of specific solutes and attribute an effect strictly to WP, the fungi must respond similarly to different types of solutes (Griffin 1977). In this study, the type and concentration of solute (sucrose or KCl) affected fungal growth. Given the differential response of the two fungi to sucrose and KCl, the effect of WP must be considered separately for each species.

Our results indicate that *G. clavigera* may have benefited from small to modest additions of solutes, and corresponding modest drops in WP, because growth was higher on amended media than on MEA. Growth of *G. clavigera* was greatest at the highest WP for each solute, when only relatively modest amounts of solute were added to the media (-0.4 and -0.8 MPa for KCl- and sucrose-amended media, respectively) (Figure 1). As WP decreased, growth of *G. clavigera* declined in a similar fashion on both types of amended media indicating a strong response of this species to WP. However, the effect of WP was not absolute because *G. clavigera* grew faster on sucrose-amended media than on KCl-amended media of similar WP. Growing in the phloem of trees, *G. clavigera* may not have a high tolerance to salts; however, it likely uses sugars as a carbon source, which may explain why growth was higher on sucrose-amended media. If *G. clavigera* assimilates sucrose, fungal growth would be stimulated on these media, offsetting some

of the adverse effects of decreased WP. Thus, changes in the concentration of solutes and available water in trees following beetle attack likely affects the growth and distribution of each fungus in the phloem, which ultimately affects its degree of interaction with the beetle. Whiting & Rizzo (1999) also found that maximum fungal growth occurred between -0.5 and -1.0 MPa on KCl- and sucrose-amended media and then fungal growth steadily decreased as WP continued to decline.

Ophiostoma montium may also have benefited from small additions of solutes because it grew slightly faster at the highest WPs on amended media than on MEA (Figure 1). However, the increased growth of *O. montium* was minimal compared to *G. clavigera*. Growth of *O. montium* at WPs below -0.4 MPa on KCl-amended media was adversely affected by decreasing WP (Figure 1). Overall, growth of *O. montium* was not greatly affected by the addition of sucrose. Many species of fungi are more sensitive to matric potential than osmotic potential; however, some wood-rotting species are extremely sensitive to osmotic potential (Griffin 1977). *Ophiostoma montium* may vary in its sensitivity to osmotic potential depending on the solute used. Closely related fungi exhibit contrasting growth rates on different types of media of the same WP (e.g., Magan & Lacey 1984; Whiting & Rizzo 1999; Whiting *et al.* 2001). This variation may be related to differences in the metabolism and nutrient requirements of different species or strains.

The differential response of *O. montium* to KCl- and sucrose-amended media confounds the interpretation of the effects of WP on the growth of this fungus. However, it indicates that the two fungal species tested in this study may differ in their resource use because growth of *O. montium* did not respond to additions of sucrose like *G. clavigera*

did. These results indicate that while *G. clavigera* and *O. montium* are known to use easily assimilable nutrients in the tree (Seifert 1993), they may actually be partitioning resources on a fine scale by using different carbon sources, or using different carbon sources at different rates. Physiological experiments conducted on a number of ophiostomatoid fungi found that all the tested species were able to use the hexose sugars glucose, fructose, mannose and galactose; however, even closely-related species varied considerably in their use of sucrose (Mathiesen-Käärrik 1960).

Klepzig *et al.* (2004) tested the growth and competitive abilities of two ophiostomatoid fungi and one Basidiomycete fungus associated with the southern pine beetle, *Dendroctonus frontalis* Zimmermann, on media amended with a combination of sucrose and KCl (together) to create WPs of 0, -5, -10, and -20 MPa. These WPs cover the range of WPs found in healthy and beetle-attacked pine trees (Klepzig *et al.* 2004). Overall, growth of each species was similar on most media (within a species); except one species grew poorly at -20 MPa. While the fungi associated with the southern pine beetle were all able to grow well on -10 MPa media amended with sucrose and KCl together (Klepzig *et al.* 2004), I found that growth of *G. clavigera* was greatly reduced by -3.3 MPa on media amended with KCl or sucrose (alone). Growth of *O. montium* was also greatly reduced at -3.3 MPa on KCl-amended media. I applied the methods of Klepzig *et al.* (2004) in a preliminary study and found that not only were *G. clavigera* and *O. montium* able to grow at -5 MPa when both solutes were used in combination, but both fungi grew fastest at -5 MPa (Bleiker & Six unpublished data). Interestingly, the lower limit of growth for wood-rotting basidiomycetes is approximately -3.9 MPa and radial growth is reduced to 50% of the maximum at -1.5 MPa (reviewed in Griffin 1977; Boddy

2000). This is similar to my results for both species on KCl-amended media. In the current study, I amended media with only one solute to test for the effect of each solute. The differential effect of sucrose and KCl on growth of *G. clavigera* and *O. montium* indicate that these solutes have physiological effects on the fungi beyond their effect on WP.

The results of this study indicate that *G. clavigera* and *O. montium* compete because both species exhibited slower growth rates when they encountered medium already colonized by the other species. The fastest growing species on each media type (*G. clavigera* in all cases except at the two lowest WPs on sucrose-amended media) was always able to colonize the most unoccupied (primary) resources before it encountered the other species. Thus, based on area colonized, the fastest growing species appears to be the superior competitor. However, both species of fungi were always able to invade medium already colonized by the other species (Figures 2-4). In fact, given the respective growth rates of each species on un-colonized media, *O. montium* was the superior competitor because it was generally more effective in colonizing media already occupied by the other species than *G. clavigera* (Figure 4). The effectiveness of *O. montium* in colonizing secondary resources may relate to its ecological role as a weakly virulent phytopathogenic species that typically follows *G. clavigera* into tree tissues (Solheim 1995; Solheim & Krokene 1998). Thus, the dominant competitor may change depending on the nature of the resource (primary or secondary) as well as with other factors, such as nutrient availability, moisture, temperature, and the presence of other species.

Although growth of both species slows when they invade medium already occupied by the other species, my results suggest that *G. clavigera* may potentially

benefit from the proximity of *O. montium* under certain conditions. Growth of *G. clavigera* was greater when it was growing near, but not directly in contact with, *O. montium* on un-amended and KCl-amended media (Figure 2). However, the difference in growth of *G. clavigera* between the two fungal treatments was relatively small. Potential benefits to *G. clavigera* of growing near, but not with, *O. montium* are unclear, but may include the production of volatiles, enzymes, or byproducts. The ability of one fungal associate to benefit from the proximity of the other associate while not competing for the same phloem resource requires further research.

This study was designed to detect the presence or absence of each species in an area but not to discern effects of WP on hyphal biomass or spore production. The few studies that have measured fungal biomass as well as WP found that fungal dry weight closely paralleled radial colony growth (reviewed in Griffin 1977). Based on the area colonized by each species in this study, competition does occur between *G. clavigera* and *O. montium* with an adverse effect being reduced growth rates when either fungus colonizes media occupied by the other species. This reduced growth rate may potentially affect the number of pupal chambers that a fungus is able to colonize, and thus, the number of beetle that it contacts. However, these negative effects may be minimal because both fungi were able to eventually colonize territory occupied by the other. Both fungi have been isolated from the phloem of attacked and co-inoculated trees (e.g., Reid *et al.* 1967; Adams & Six 2007; Rice *et al.* 2007; Chapter 3). Thus, prior to brood emergence from the natal host when teneral adults are pre-emergence feeding and acquiring spores for dispersal, abiotic factors, such as temperature (Six & Bentz 2007), nutrient availability, or moisture, may be more influential in determining beetle-fungal

interactions than interspecific fungal competition. In fact, in Chapter 3 I did not find a negative correlation between the percent of phloem colonized by *G. clavigera* or *O. montium* alone in beetle-attacked trees as would be expected with competition. The only indication of competition occurred towards the end of the one-year life cycle of the beetle when resources were likely limited in the tree. At that time, the amount of phloem colonized by both fungi together decreased and there was a corresponding increase in the amount of phloem from which no fungi were isolated. In Chapter 3, I hypothesized that resource use is greater in phloem colonized by both species compared to when only one species colonizes the phloem. Thus, resources may fall more quickly below the minimum threshold required to support the fungi in phloem colonized by both fungi together compared to phloem colonized by only one fungus. This would suggest that competition between the fungi is likely a result of exploitation of a shared resource rather than from direct combative interactions between the species. However, interference competition, such as direct interactions between hyphae, cannot be ruled out.

The differential response of the fungi to the two solutes confounds the effect of WP on *O. montium*; however, it revealed that *G. clavigera* and *O. montium* may partition nutrient resources on a fine scale. This may explain, at least in part, why Chapter 3 found a reduction in the percent of phloem colonized by *O. montium* only towards the end of the one-year life cycle of the beetle, but not a reduction in the amount of phloem colonized by *G. clavigera* only. Depletion of a nutrient used primarily by *O. montium* before a nutrient that is used primarily by *G. clavigera* could account for the decrease in the percent of phloem colonized by *O. montium* only towards the end of the beetle's life cycle.

The ophiostomatoid fungal associates of the mountain pine beetle depend on the beetle for transport to a suitable habitat (a beetle-attacked tree) - a spatially and temporally limited resource. Both fungi arrive and disperse from their habitat at the same time on their 'beetle taxis'. The fungi are unit-restricted dispersers on a discontinuous resource and when resources in their current habitat are depleted they are extirpated. Between their arrival and departure, the fungi must rapidly colonize phloem in order to sporulate in as many pupal chambers as possible to maximize production of spores, and acquisition and dispersal by beetles to future habitats. The results of this study indicate that competition occurs between *C. clavigera* and *O. montium* on artificial media; however, the fungi are also able to coexist without competitive exclusion. The differential effect of KCl and sucrose on the growth of *G. clavigera* and *O. montium* suggests that these fungi may be able to partition certain resources on a fine scale, which could promote their co-existence. In addition, *G. clavigera* dominates primary resources, but *O. montium* is more effective at capturing secondary resources given the relative growth rates of the two fungi. However, the interaction between the two fungi takes place in nature in a complex environment: conditions (e.g., moisture, nutrients, the presence of other species) change rapidly in trees following attack and abiotic factors, such as temperature, may also affect interactions between the fungi. Thus, fine scale resource partitioning, differences in primary and secondary resource capture abilities, and the non-equilibrium dynamics in the attacked tree over time, could all act to promote the co-existence of two unit-restricted dispersers on a discontinuous resource.

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Table 1. Theoretical water potential (WP) of amended media treatments based on the osmotic potential of the added solute and the actual mean (SD) WP as measured with a Dewpoint PotentialMeter at day 0 and day 30.

Theoretical WP (MPa)	Actual mean (SD) WP of amended media (MPa) [†]			
	MEA amended with KCl		MEA amended with sucrose	
	Day 0 [‡]	Day 30 [‡]	Day 0	Day 30
0.00	-0.41 (0.09)	--	-0.80 (0.10)	--
-0.25	-0.56 (0.08)	--	-0.99 (0.16)	--
-0.50	-0.82 (0.09)	--	-1.10 (0.07)	--
-1.00	-1.37 (0.11)	-1.22 (0.06)	-1.47 (0.06)	--
-2.00	-2.24 (0.02)	-2.01 (0.02)	-2.69 (0.19)	-2.44 (0.25)
-3.00	-3.28 (0.24)	-3.41 (0.14)	-3.32 (0.11)	-3.56 (0.18)

[†] WP of un-amended MEA was -0.13 (0.06) MPa at day 0 and -0.16 (0.07) MPa at day 30.

[‡] Sample sizes are $n=4$ Petri plates for each WP at day 0 and $n=3$ Petri plates for each WP at day 30.

Table 2. Number of days required for *G. clavigera* and *O. montium* to fully colonize media in five 9 cm diameter Petri dishes with a range of water potentials (WPs) and the corresponding treatment sample times for the staggered, overlapping inoculation experiment.

Media WP and type	Time (days)		Treatment sample times (experiment day)			
	C [†]	M [†]	M(+C) [‡]		C(+M) [‡]	
			Time 1	Time 2	Time 1	Time 2
Un-amended MEA	7	8	17	24	18	26
-0.4 KCl	7	8	17	24	18	26
-0.6 KCl	9	9	19	28	19	28
-0.8 KCl	10	11	20	30	21	32
-1.4 KCl	12	20	22	34	30	--
-0.8 sucrose	7	8	17	24	18	26
-1.0 sucrose	7	9	17	24	19	28
-1.1 sucrose	8	9	18	26	19	28
-1.5 sucrose	9	10	19	28	20	30
-2.7 sucrose	11	9	21	32	19	28
-3.3 sucrose	20	10	30	--	20	30

[†] Fungal codes: C = *G. clavigera*; and M = *O. montium*.

[‡] Fungal treatment codes: M(+C) = *O. montium* colonized media for 10 days before *G. clavigera* was inoculated; C(+M) = *G. clavigera* colonized media for 10 days before *O. montium* was inoculated.

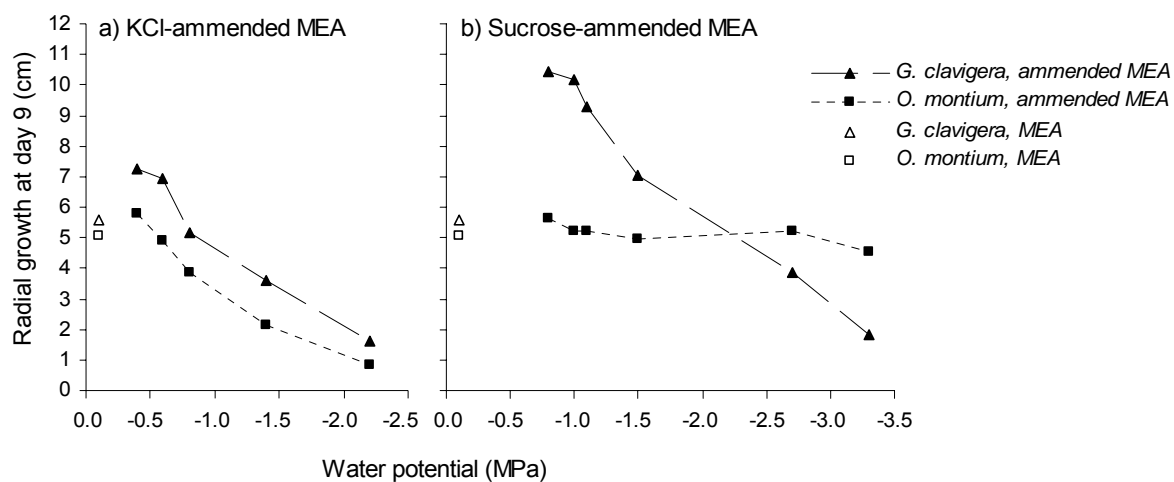


Figure 1. Mean radial growth of *G. clavigera* and *O. montium* (growing alone) after 9 days on 2% malt extract agar (MEA) and MEA amended with (a) KCl or (b) sucrose to obtain a range of water potentials. SEs are not shown because they were very small and mostly obscured by the symbols.

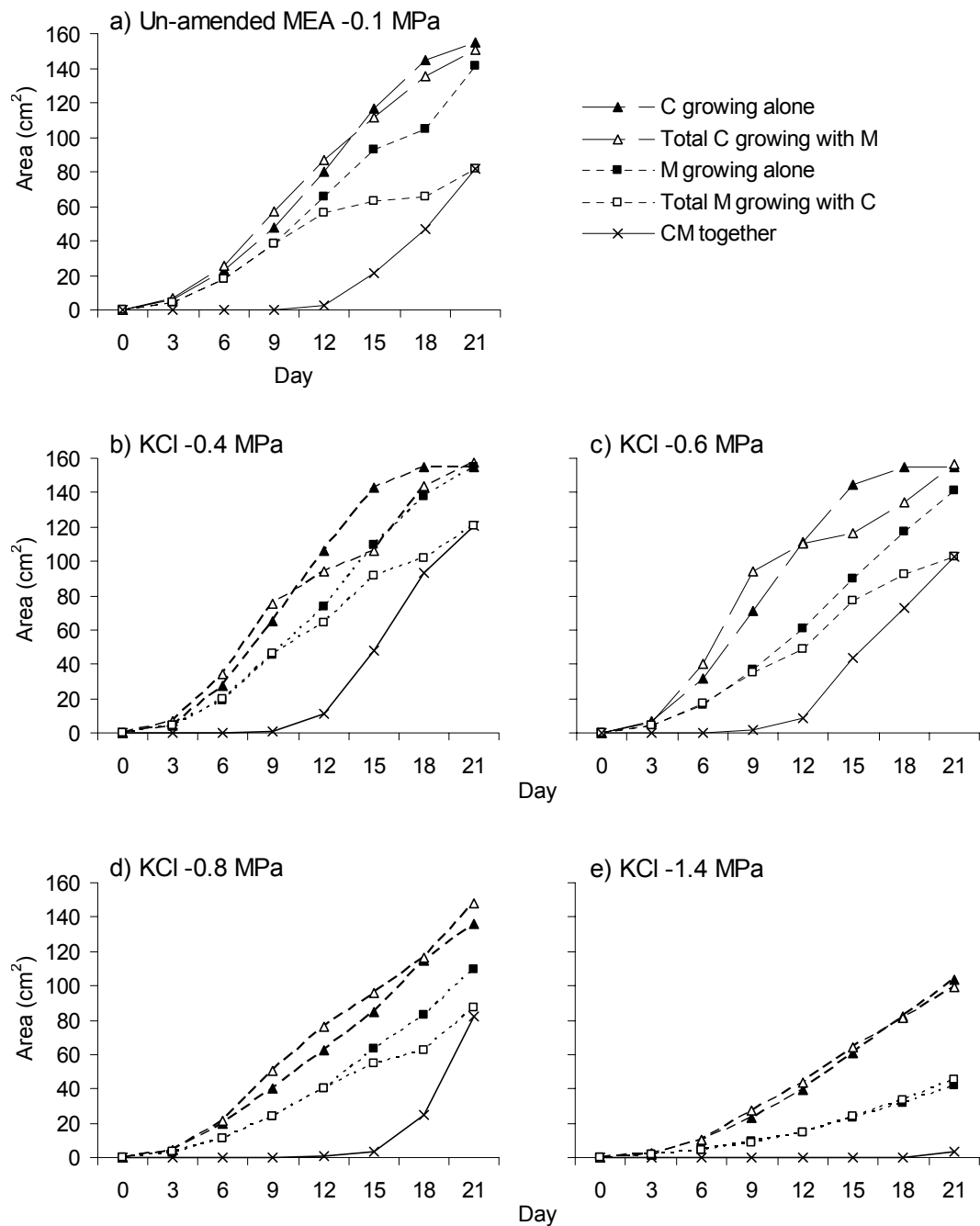


Figure 2. Area colonized by *G. clavigera* and *O. montium* growing alone and with each other on (a) 2% malt extract agar (MEA) and (b) MEA amended with KCl to create a range of water potentials. SEs are not shown because they were very small and mostly obscured by the symbols.

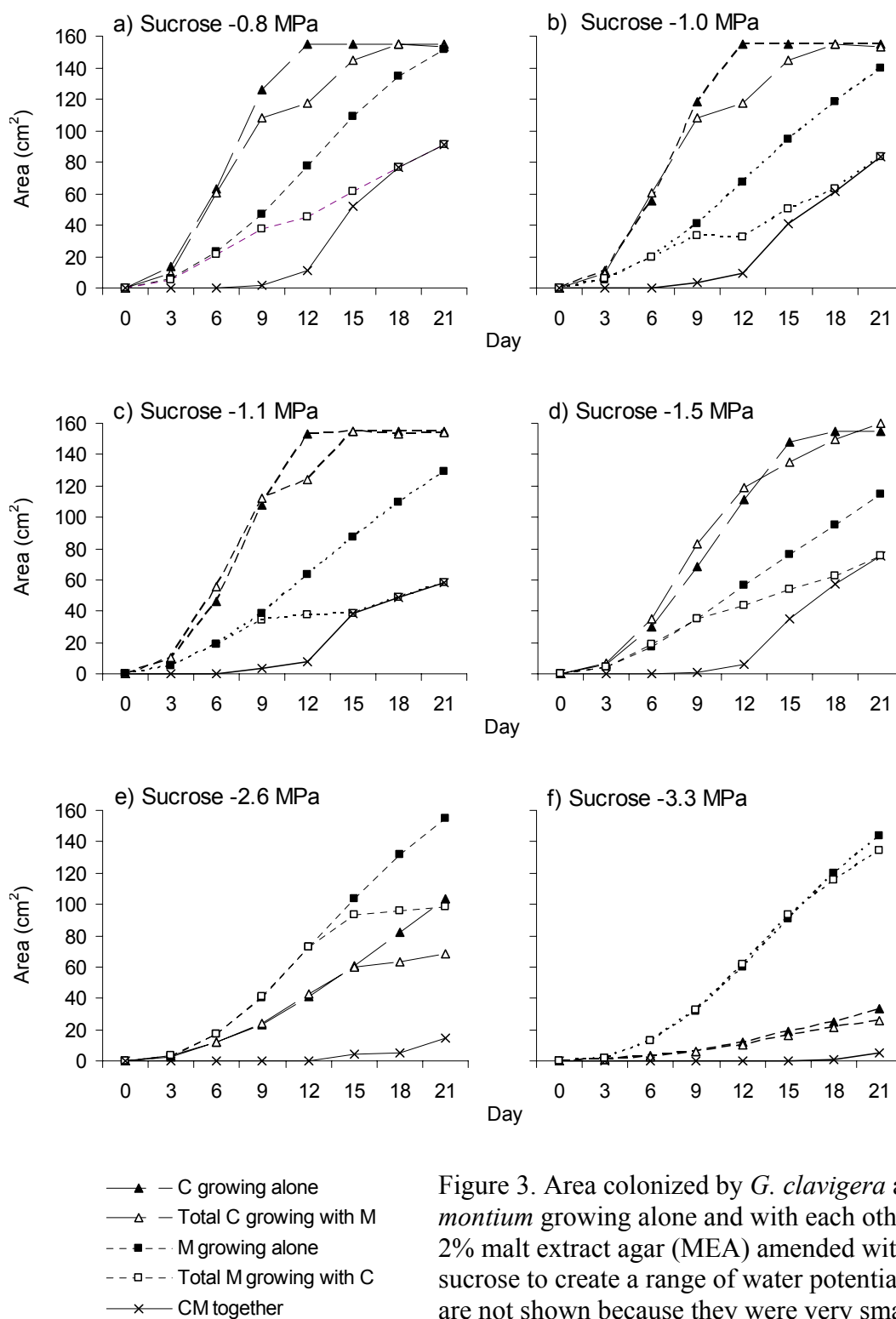


Figure 3. Area colonized by *G. clavigera* and *O. montium* growing alone and with each other on 2% malt extract agar (MEA) amended with sucrose to create a range of water potentials. SEs are not shown because they were very small and mostly obscured by the symbols.

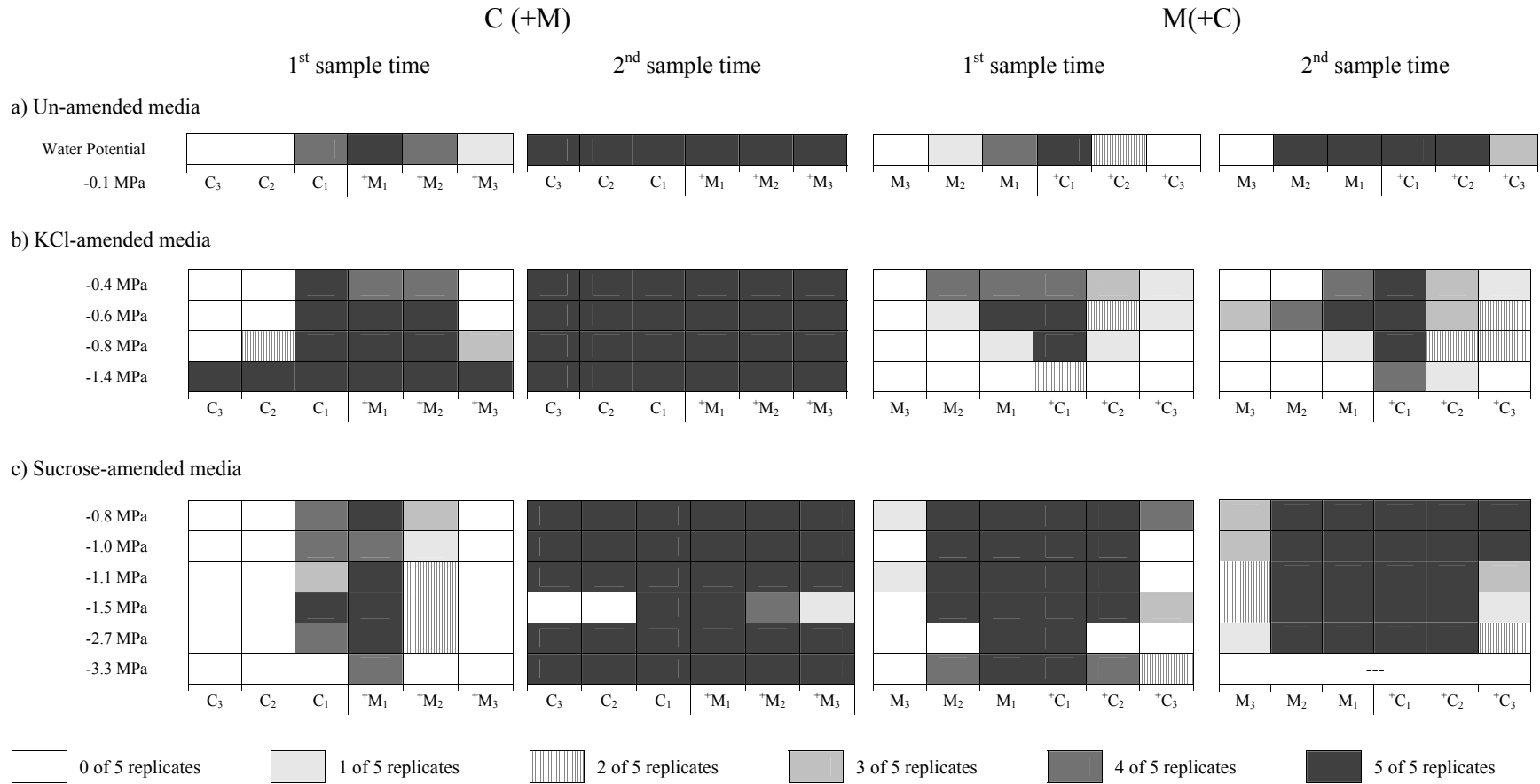


Figure 4. Number of times *O. montium* (M) and *G. clavigera* (C) were isolated from (a) un-amended media, (b) KCl-amended media, (c) sucrose-amended media at six sample locations from five Petri dishes per treatment. For each treatment, one species was inoculated into the center of the dish on day 0 and the second species was inoculated immediately adjacent to it on day 10. Fungal treatments are coded as follows: M(+C) = *O. montium* colonized the medium for 10 d before *G. clavigera* was inoculated; C(+M) = *G. clavigera* colonized medium for 10 d before *O. montium* was inoculated. Samples were taken from mirrored locations on either side of the inoculation plugs (C or M) and are coded as follows: 1 - immediately adjacent to the fungal inoculation plug; 2 - halfway between the fungal inoculation plug and the edge of the Petri dish; 3 - adjacent to the edge of the Petri dish.

Chapter 3

Competition and coexistence in a multi-partner mutualism: Interactions between two fungal symbionts of the mountain pine beetle in beetle-attacked trees

Abstract

Despite overlap in niches, two fungal symbionts of the mountain pine beetle (MPB) (*Dendroctonus ponderosae*), *Grosmannia clavigera* and *Ophiostoma montium*, appear to coexist with one another and their bark beetle host in the phloem of trees. I sampled the percent of phloem colonized by fungi four times over one year to investigate the nature of the interaction between these two fungi and to determine how changing conditions in the tree (e.g., moisture) affect the interaction. High phloem moisture appeared to inhibit fungal growth shortly after beetle attack; however, after one year phloem moisture likely inhibited fungal growth and survival. Both fungi colonized phloem at similar rates; however, *G. clavigera* colonized a disproportionately larger amount of phloem than *O. montium* considering their relative prevalence in the beetle population. There was no inverse relationship between the percent of phloem colonized by *G. clavigera* and *O. montium* that would indicate competition between the species. However, the percent of phloem colonized by *G. clavigera* and *O. montium* together decreased after one year, while the percent of phloem from which no fungi were isolated increased. A reduction in phloem colonized by fungi at this time may have significant impacts on both beetles and fungi. New adult beetles preferentially feed on fungal spores prior to emergence, and the fungi must sporulate in pupal chambers if they are to be acquired by beetles and transported to a new tree. These results indicate that exploitation competition was prevalent after a year when the fungi co-occurred in the phloem, but I found no evidence of strong interference competition. Each species also maintained an exclusive area, which may promote coexistence of species with similar resource use.

Introduction

The effect of interspecies interactions in shaping ecological communities is well known. Closely related species that co-occur in a habitat and have similar resource requirements may be expected to compete with each other. Competition occurs when one species negatively affects another by consuming a common limited resource (exploitation or indirect competition) or when one species controls access to a limited resource (interference or direct competition) (Keddy 1989). Competition often results in competitive exclusion with the victor being the competitor that can maintain itself on the lowest level of the common resource (Gause 1934). Interspecies interactions research has focused on competition between plants growing at high densities and animals competing for the same resource; comparatively less is known about interspecific interactions involving fungi, including competition (Shearer 1995).

Interactions among fungi may affect other organisms in the community through multi-trophic interactions. For example, many phloeophagous bark beetles (Coleoptera: Curculionidae, Scolytinae) have associations with ophiostomatoid fungi (Ascomycotina) that are considered mutualistic (Six 2003a; Harrington 2005). The fungi are transported by the beetles to otherwise inaccessible habitats. The beetles may receive one or more benefits from their fungal associates, such as nutritional supplementation, conditioning of host tree tissues for brood development (e.g., chemical changes), or aid in overwhelming tree defenses (reviewed in Paine et al. 1997). Bark beetle-fungal associations are not always mutualisms and can also include commensalisms and antagonisms, in addition to being context-dependent (Barras 1973; Six 2003a; Klepzig & Six 2004).

Mutualistic and antagonistic interactions with fungi may affect beetle fitness and potentially influence beetle population dynamics (Bridges 1985; Six & Paine 1998; Lombardero et al. 2003; Hofstetter et al. 2006a, 2006b; Bleiker & Six in press). For example, the southern pine beetle (*Dendroctonus frontalis* Zimm.) receives nutritional benefits from two fungal associates, although one species provides greater benefit to the beetle than does the other (Bridges 1985; Goldhammer *et al.*, 1990; Coppedge *et al.*, 1995). A third fungal associate of the southern pine beetle that is also associated with phoretic mites adversely affects brood survival (Barras 1973). Because fungal associates may differentially affect beetle fitness, interactions among the fungi (e.g., competition) may indirectly affect beetle fitness by influencing the distribution of fungi within a tree and their availability to beetles.

The fungal associates of bark beetles share the same resources within a tree: a limited substrate and easily assimilable nutrients present in the phloem (Seifert 1993). Species co-occurring on the same resources are likely to compete and the species that can live on the lowest level of a resource is predicted to exclude its competitors over time (competitive exclusion). Laboratory studies have demonstrated that the fungal associates of bark beetles can compete and that the dominant competitor usually is the species with the fastest intrinsic growth rate (Klepzig & Wilkens 1997; Chapter 2). However, growth of bark beetle-associated fungi may also be affected by other factors, such as the water potential (Klepzig *et al.* 2004; Chapter 2) and phloem chemistry (Shrimpton & Whitney 1968; Hofstetter et al. 2005) of the tree, and the presence of other microorganisms (e.g., yeasts and bacteria) (Graham 1967; Adams & Six in review). Previous studies examining competitive interactions among bark beetle fungal associates have been conducted on

artificial media in the laboratory under controlled conditions. However, in trees, conditions change radically over the life cycles of both the beetles and fungi, which could potentially affect the outcome of fungal interactions. Therefore, field studies are needed to determine how fungi actually grow and interact in trees over time.

I examined interactions between two ophiostomatoid fungi, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield (previously *Ophiostoma clavigerum*) and *Ophiostoma montium* (Rumbold) von Arx in trees colonized by the mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins). Numerous microorganisms are associated with the MPB; however, *G. clavigera* and *O. montium* are the only filamentous fungi consistently isolated from its mycangia (structures of the integument specialized for transporting fungi) (e.g., Whitney & Farris 1970; Whitney 1971; Six 2003b; Bleiker & Six in press). The fungi are inoculated into tree tissues by parent beetles as they construct egg galleries in the phloem. Fungal spores are transported on the exoskeleton of MPB adults as well as in mycangia, which are located on the maxillary cardines (Whitney & Farris 1970; Six 2003a). Developing larvae ingest phloem and fungal hyphae as they construct feeding galleries and teneral (young, sexually immature) adults often feed on spores that commonly line pupal chambers prior to emergence (Leach et al. 1934; Whitney 1971; Six & Paine 1998; Adams & Six 2007). Fungal spores are acquired on the exoskeleton and in mycangia of teneral adults during pre-emergence feeding (Whitney & Farris 1970; Six & Paine 1998).

Both fungi appear to enhance beetle fitness through nutritional supplementation, but the two fungi are not equal in their effects. Evidence indicates that while *G. clavigera* confers the greatest fitness benefits to the MPB, having *O. montium* as an associate is

better than having no fungal associate (Six & Paine 1998; Bleiker & Six in press). *G. clavigera* and *O. montium* differ in their environmental tolerances and having two mutualists may reduce the chance that the beetle is left without the benefits of a partner if the environment is unfavorable for one associate (Six & Bentz 2007). Larval preference for phloem colonized by both fungi together compared to phloem colonized by only one fungus, indicates that the two fungi may provide complimentary benefits (Bleiker & Six in press). Given the potential importance of *G. clavigera* and *O. montium* in the diet of the MPB and their effects on beetle fitness, fungal growth in trees and interactions between the fungi may have significant implications for developing beetles.

In a previous study conducted on artificial media, I observed reduced growth of both *G. clavigera* and *O. montium* when they were growing together compared to when they were growing alone, indicating interspecific competition (Chapter 2). I also investigated the effects of water potential on the growth of both species by amending media with potassium chloride or sucrose to create a range of water potentials. On potassium chloride-amended media, growth of both species decreased as water potential decreased (-0.4 through -3.3 MPa). On sucrose-amended media, growth of *G. clavigera* also declined as water potential decreased; however, growth of *O. montium* remained relatively constant regardless of water potential. The solute used to amend the media confounded the effect of water potential, negating inferences on how changes in phloem moisture in beetle-attacked trees might affect interactions between *G. clavigera* and *O. montium*.

It is difficult to infer how the fungi may interact in trees from laboratory experiments, even without the many confounding factors associated with the different chemical

makeup of tree phloem and the rapidly changing nature of the tree after beetle attack. Therefore, I examined colonization rates and the relative prevalence of *G. clavigera* and *O. montium* in trees attacked by the MPB in the field over the one-year life cycle of the beetle. I also correlated phloem moisture, which changes dramatically in attacked trees and may affect fungal growth, to the relative prevalence of the two fungi in trees.

Materials and Methods

The prevalence of *G. clavigera* and *O. montium* in the phloem of lodgepole pine trees (*Pinus contorta* var. *latifolia* Dougl. Ex Loud.) successfully attacked by the MPB was sampled over the one-year life cycle of the beetle. In early June 2003, prior to the beetle's flight, a small stand of mostly healthy (un-attacked) mature lodgepole pine trees was selected on the DeBorgia Divide outside of Thompson Falls in western Montana (47°29'0"N and 115°15'11"W; elev. 1,510 m). The MPB population in the area had been at outbreak levels for a number of years and MPB-caused mortality was evident in most stands of mature lodgepole pine in the area. Given the high beetle pressure and the dwindling supply of host trees in the area, I predicted that the MPB would colonize a high percentage of the remaining healthy trees in this stand in summer 2003.

Anticipating attack by the MPB, 60 mature lodgepole pine trees were numbered and monitored for boring dust (indicating successful MPB attack) starting on 10 June 2003. I checked each tree every 3 to 4 d and the day of attack (time zero, T₀) was recorded as the date when the tree was first observed to be mass attacked (i.e., high density of attacks initiated on the lower bole). Over the course of the summer, 43 of the 60 trees were mass attacked by the MPB. Fifteen trees, representing the full range of attack dates, were

selected for sampling. Wildfires in the area temporarily blocked access to the site from 12 to 26 August; however, observations made on 26 August (and after) revealed that no successful attacks were initiated after 11 August. Trees were grouped into three categories based on time of mass attack: early season-attacked trees (2 trees, attacked on 26 June); mid-season attacked trees (8 trees attacked between 10 July and 28 July); and late season-attacked trees, (5 trees attacked on 11 August). I sampled each tree at 4 different times: 2-4 wks after attack (T1, eggs and early instar larvae (1st or 2nd instars) were the most common life stage observed); 9-11 wks after attack prior to over-wintering (T2, late instar larvae (3rd or 4th instars) were the most common life stage observed); 42-47 wks after attack (T3, prepupae or pupae were the most common life stage observed); and 52-57 wks after attack just prior to brood emergence (T4, teneral adults were the most common life stage observed).

At each sample time, I removed a 15 x 15 cm square bark sample at 4 target heights (1, 2, 3, and 4 m) from a randomly chosen aspect on the bole of each of the 15 trees for a total of 240 bark samples. Bark samples were staggered just above or below the target height in order to accommodate the 4 samples taken over time. Prior to removing the bark sample, the rough outer bark flakes were rubbed off and the area was sprayed with 95% ethyl-alcohol. Bark samples were removed with a chisel that had been sprayed with 95% ethyl-alcohol. The exposed sapwood was sprayed with pruning seal to reduce moisture loss. Each bark sample was placed in a plastic bag that was sealed and stored in a cooler or refrigerator for up to 2 d before isolations were made. Using sterile technique, 16 phloem plugs were removed from each bark sample on a 4 X 4 cm grid for a total of 3,840 phloem samples over the course of the study. Samples were removed with a 3 mm

sterile diameter cork borer just below the phloem-cambium surface in order to exclude any contaminants acquired while handling the sample.

Phloem samples were placed on 2% malt extract agar (MEA) amended with 100 ppm cyclohexamide (Harrington 1981). Cyclohexamide allows for the growth of *Ophiostoma* and *Grosmannia* species, while inhibiting the growth of ubiquitous, non-symbiotic fungi from the environment (e.g., *Trichoderma* and *Penicillium* spp.), which can quickly overrun cultures. Cultures were grown at approximately 22°C for a minimum of 8 weeks before colonies were identified using morphological characteristics (hyphae, asexual and sexual structures) (Upadhyay 1981). Pieces of autoclaved pine twigs were added to the cultures after the initial identifications were made because the fungi, especially *O. montium*, sporulate more readily on pine twigs than on artificial media, greatly facilitating identification. The cultures were re-examined after an additional 4 to 6 weeks. Most cultures of ophiostomatoid fungi also contained yeasts and likely bacteria. Because it was difficult to determine if any cultures containing *G. clavigera* and/or *O. montium* were truly free of other microorganisms and other microorganisms were not the focus of the study, I classified cultures with respect to the ophiostomatoid fungi. I calculated the percent of phloem colonized by *G. clavigera* only (C only), *O. montium* only (M only), both fungi growing together (CM), as well as the percent of phloem colonized by ophiostomatoid fungi (total Oph.) (previous three categories summed). The percent of cultures from which only yeasts were isolated (yeast only) (no ophiostomatoid fungi present) and from which no microorganisms were isolated (i.e., no live microorganisms in sample) was also calculated.

Phloem moisture was measured at each of the four sampling times (T1, T2, T3, and T4), as well as on the sample date when mass attacks were first identified (T0). A 2.5 cm diameter arch punch was used to remove a phloem plug at 1.5, 2.5, and 3.5 m on the tree bole. The phloem plugs were immediately placed in small, individual plastic bags and transported on ice to the laboratory. The average percent dry weight phloem moisture content was calculated for each tree using the oven dry weight method and the formula: $[(\text{wet weight} - \text{dry weight}) / \text{dry weight}] \times 100$. Phloem samples were dried for 48 h at 60°C prior to taking the dry weight.

Phloem moisture was also measured on un-attacked (control) trees using the methods described above for attacked trees. Most of the trees originally selected as controls were subsequently attacked by beetles and are hereafter referred to as ‘eventually attacked trees’. New, un-attacked control trees were selected to replace the controls that were attacked; however, many of these trees were also subsequently attacked. As a result, phloem moisture was only measured in 6 true control trees (referred to as ‘never attacked’); one tree was sampled at 3 different sample times over the summer, one tree was sampled twice, and the other 4 trees were only sampled at one sample time each. These 9 measurements of phloem moisture for trees that were never attacked spanned the entire attack period from 26 June to 11 August 2003. Phloem moisture was also measured in 10 trees that were eventually attacked; all trees in this category were only sampled once (because they were mass attacked by the next sample time). Sample times again spanned the entire attack period.

Data analysis. In order to meet the assumptions of normality, the arcsine (or angular) transformation (Sokal & Rohlf 2000) was applied to the percent of phloem colonized by

fungi. Transformed data were used in all statistical tests. Back transformed means and Least Squares (LS) means are reported and used in graphs. Upper and lower standard errors (SEs) were calculated from back transformed confidence intervals (± 1 SE), which were calculated from the transformed means. Phloem moisture data did not require transformation to meet the assumptions of the analyses.

A repeated measures multivariate analyses of variance (MANOVA) was used to test for variation in the percent of phloem colonized by *G. clavigera* and *O. montium* among sample heights (1, 2, 3, and 4 m) and fungi isolated (C only, M only, and CM). All interactions, including those with time, were included in the analysis. Because sample height and interactions involving sample height were not significant (see Results), data for the samples were summed by tree so that sample height could be removed from the analyses. This decreased the number of factors and excluded four-way interactions in the analysis and provided a better estimate of the percent of phloem colonized by the fungi at each sample time (now calculated from 64 phloem samples per tree). A repeated measures MANOVA was then used to test for the effects of time of attack (early-, mid-, and late-season attacked trees), fungal species isolated, and their interaction on the percent of phloem colonized by *G. clavigera* and *O. montium*. All interactions, including those with time, were included in the analysis. Significant multivariate results were followed by univariate ANOVAs conducted using a General Linear Model (GLM) approach. Significant *F* tests were followed by Tukey-Kramer's Honestly Significant Difference (HSD) test, which is a conservative post-hoc test appropriate for uneven sample sizes (Sokal & Rohlf 2000).

The Pearson product-moment correlation coefficient (Pearson's r) was used to test for a correlation between the percent of phloem colonized by *G. clavigera* only and *O. montium* only at the same sample time. The percent of phloem colonized by both fungi growing together was also correlated with the percent of phloem colonized by *G. clavigera* only and *O. montium* only at each sample time.

To determine how phloem moisture in un-attacked trees varied over the course of a season, a two-way ANOVA was used to test for variation in phloem moisture with sample date (7 dates over the course of the summer). Phloem moisture measurements from control trees (never attacked or eventually attacked) and attacked trees at T0 were included in the analysis. Tree group (never attacked, eventually attacked, or attacked at T0) was included as a second factor in the ANOVA. The effects of time of attack (early-, mid-, and late-season attacked trees), fungal species, and their interaction on phloem moisture in the 15 attacked trees was examined using a repeated measures MANOVA. Significant multivariate results were followed by ANOVAs conducted using a GLM approach. Significant F tests were followed by Tukey-Kramer's HSD test.

Pearson's r was used to test for an association between phloem moisture at one sample time and phloem moisture at the previous sample time in attacked trees. Pearson's r was also used to test for a relationship between the amount of phloem colonized by *G. clavigera* only, *O. montium* only, or both fungi together and phloem moisture at the same and preceding sample time.

Significance was set at $P \leq 0.05$ and all analyses were conducted using JMPTM 5.1 (SAS Institute, Cary, North Carolina, USA)

Results

Attack Period

MPB initiated mass attacks on 43 of the 60 mature lodgepole pine trees that were monitored during the summer of 2003 (Figure 1a). MPB mass attacked trees between 23 June and 11 August; however, most new attacks were found on 23 and 28 July (11 and 14 trees, respectively) (Figure 1a). Thus, the peak attack period for the MPB likely occurred around this time (Figure 1a).

Phloem moisture

Unattacked trees and attacked trees at T0 (day of mass attack). Trees that were not attacked during the 2003 season had higher phloem moisture than trees that were sampled prior to being attacked but which were eventually attacked later in the season, or trees that were sampled in the very initial stage of mass attack (T0) ($F_{2,25}=5.92$, $P=0.008$) (Figure 2). Phloem moisture decreased in all trees during the last two weeks of July ($F_{6,25}=8.61$, $P<0.0001$) (Figure 1b). Phloem moisture was higher in trees sampled on or before 14 July 2003 than in trees sampled on or after 28 July 2003; trees sampled on 18 and 23 July were not different from trees sampled earlier or later in the season (Figure 1b).

Attacked trees. Phloem moisture of trees mass attacked by the MPB varied with sample date ($F_{4,9}=603.93$, $P<0.0001$), but not with time of mass attack (trees attacked early-, mid-, or late-season) ($F_{2,12}=0.17$, $P=0.85$) (Figure 3). Overall, phloem moisture decreased rapidly after the initial mass attack, dropping over 40% in the first 2-4 weeks (between T0 and T1) (Figure 3). Although phloem moisture decreased rapidly in all trees following attack, differences among early-, mid-, and late-season attacked trees varied

over time (significant interaction, $\lambda_{8,18}=0.07$, $P=0.0008$) (Figure 3). Late-season attacked trees had lower phloem moisture when mass attacks were first initiated (T0) compared to trees that were attacked early- or mid-season ($F_{2,36}=19.61$, $P<0.0002$) (Figure 3), likely because phloem moisture decreases in trees over the summer. At sample times T1 and T2, phloem moisture did not vary with time of attack ($F_{2,36}=2.36$, $P=0.14$ and $F_{2,36}=0.77$, $P=0.48$, respectively). At T3, time of attack was significant ($F_{2,36}=3.88$, $P=0.05$): Phloem moisture was 25% in early-season attacked trees, 32% in mid-season attacked trees, and only 47% in late-season attacked trees; however, no pairwise comparisons were statistically different. At the last sample time, there was little variation in phloem moisture and it did not vary among trees attacked early-, mid-, and late-season ($F_{2,36}=2.71$, $P=0.11$).

Phloem moisture at each of the four sample times was not correlated with the previous sample time (T0-T1, $r=0.38$, $P=0.17$; T1-T2, $r=0.34$, $P=0.22$; T2-T3, $r=0.39$, $P=0.15$; T3-T4, $r=-0.02$, $P=0.95$).

Fungal colonization in attacked trees

Grosmannia clavigera, *O. montium*, and yeasts were the most common microorganisms isolated from the phloem of MPB-attacked trees. The percent of phloem colonized by these fungi was similar in early-, mid-, and late-season attacked trees (Figure 4). The percent of phloem colonized by *G. clavigera* and/or *O. montium* is described below with the results of the repeated measures MANOVA. Yeasts were the only microorganisms isolated from approximately 13% of the phloem samples at T1; however, the number of samples containing only yeasts dropped to near 0% at later sample dates when much of the phloem was colonized by ophiostomatoid fungi (in

addition to yeasts much of the time) (Figure 4). No microorganisms were isolated from over 50% of phloem samples at T1 (Figure 4). However, the percent of samples containing only un-colonized phloem dropped dramatically by T2 when most phloem was already colonized by ophiostomatoid fungi.

The percent of phloem colonized by the ophiostomatoid fungal associates of the MPB did not vary with sample height on the bole of the tree ($F_{3,168}=0.07$, $P=0.97$) and there was no sample height by fungus interaction ($F_{6,168}=1.06$, $P=0.39$). Thus, isolations made from the 4 bark samples were combined by tree for each sample time and the percent of phloem colonized was calculated from a total of 64 phloem samples per tree at each sample time. Fungus species isolated and sample time were significant factors in this analysis (results for these factors are reported below for the test using data summarized at the tree level).

The percent of phloem colonized by ophiostomatoid fungi varied over time ($F_{3,34}=36.67$, $P<0.0001$) (Figure 5). The percent of phloem colonized by ophiostomatoid fungi increased sharply between T1 and T2, remained relatively constant between T2 and T3, and decreased between T3 and T4 (Figure 5). The percent of phloem colonized by ophiostomatoid fungi did not vary with time of attack ($F_{2,36}=1.31$, $P=0.28$), but did vary by fungus ($F_{2,36}=13.96$, $P<0.0001$) (Figure 5). The interaction between sample time and time of attack did not affect the percent of phloem colonized by ophiostomatoid fungi ($F_{6,68}=1.50$, $P=0.19$). The interaction between fungus and time of attack had a significant effect ($\lambda_{6,68}=6.25$, $P<0.0001$), and the three-way interaction among sample time, time of attack, and fungus on phloem colonized by ophiostomatoid fungi was marginally significant ($\lambda_{12,90}=0.57$, $P=0.06$). At T1, the percent of phloem colonized did not vary

with fungus ($F_{2,36}=0.42$, $P=0.66$) and there was no interaction between time of attack and fungus ($F_{4,36}=1.96$, $P=0.12$). At T2, there was more phloem colonized by hyphae of both fungi together, or *O. montium* only, compared to *G. clavigera* only. The fungus by time of attack interaction approached significance ($F_{4,36}=2.41$, $P=0.07$), likely due to the relatively large percentage of phloem colonized by both fungi in late-season attacked trees (Figure 5c). At T3, there was more phloem colonized by both fungi together than by either fungus alone ($F_{2,36}=23.94$, $P<0.0001$); however, this was only true for early- and late-season attacked trees (significant interaction $F_{4,36}=6.12$, $P=0.0007$) (Figure 5). At T4, the percent of phloem colonized did not vary with fungus or the fungus by time of mass attack interaction ($F_{2,36}=0.23$, $P=0.79$ and $F_{4,36}=0.15$, $P=0.96$, respectively).

There were no significant correlations between the percent of phloem colonized by *G. clavigera* only and *O. montium* only at any sample time (Table 1). Similarly, there were no significant correlations between the percent of phloem colonized by *G. clavigera* only and both fungi together at any sample time (Table 1). The percent of phloem colonized by *O. montium* only and both fungi together was negatively correlated at T2 and T3 (Table 1).

Phloem moisture and fungal colonization

The percent of phloem colonized by *G. clavigera* only was not correlated with phloem moisture at the same or preceding sample time (Table 2). Correlations between the percent of phloem colonized by *O. montium* only and phloem moisture were only significant at T1 when higher phloem moisture corresponded to a decrease in the percent of phloem colonized by *O. montium* (Table 2). The only significant correlation between phloem moisture and the percent of phloem colonized by both *G. clavigera* and *O.*

montium together was when the percent of phloem colonized at T4 was positively correlated with phloem moisture at T3 (Table 2). There were two significant correlations between the total percent of phloem colonized by ophiostomatoid fungal associates of the MPB and phloem moisture. These were a negative correlation between phloem colonized at T2 and phloem moisture at T1 and a positive correlation between the total percent of phloem colonized at T4 and phloem moisture at T3 (Table 2).

Discussion

The multiple fungal associates of bark beetles coexist in a highly variable environment, which changes rapidly throughout the year-long developmental period of the insect. Each fungus must not only survive in this rapidly changing habitat, but also sporulate in pupal chambers of the beetles at the time when new adults are pre-emergence feeding so that spores can be acquired in the mycangia and disseminated to the next habitat (tree). By colonizing more phloem, a fungus will be able to sporulate in more pupal chambers and be acquired by more beetles, increasing the number of spores that are transmitted to new trees by beetles. Because spores are not acquired by new adults until the end of their one-year life cycle (after pupation), a fungus must not only colonize as much phloem as possible under rapidly changing conditions in the tree, but it must also live long enough in the phloem so that it can sporulate when adults are eclosing from the pupal stage. Changes within a tree over time (e.g., moisture, nutrient levels, and temperature) may have significant effects on fungal growth and survival. Thus, the distribution of *G. clavigera* and *O. montium* within a tree, as well as interactions between them, may change over time. Given the differential effects of *G. clavigera* and *O.*

montium on MPB fitness (Six & Paine 1998; Bleiker & Six in press), the distribution of each fungus, as well as interactions between the fungi, may ultimately impact the MPB by affecting how many beetles are exposed to each fungus.

O. montium and *G. clavigera* colonized the phloem of trees at similar rates, except for the two early-attacked trees where *O. montium* colonized the phloem faster than *G. clavigera* (Figure 5). However, considering the relative inoculation rates of the two fungi by attacking beetles, *G. clavigera* colonized a disproportionate amount of phloem and may have actually colonized the phloem faster than *O. montium*. In summer 2003, the year that the trees were attacked, 88% of MPBs caught in flight traps at the site carried only *O. montium*, while 11% carried only *G. clavigera*; less than 1% of beetles carried both fungi (Thompson Falls site, Six & Bentz 2007). Assuming that the fungal complement carried by dispersing beetles at the site reflects inoculation rates for each species of fungus into trees, *O. montium* may have initially colonized a larger proportion of phloem because it was inoculated into trees at a rate eight times higher than *G. clavigera*. Based on the proportion of dispersing beetles carrying each fungus, *G. clavigera* appears to have colonized a disproportionately large amount of phloem compared to *O. montium*. *G. clavigera* is considered to be a more virulent pathogen with a higher tolerance for low oxygen conditions than *O. montium*, and thus may be better adapted for growing in living, defended tree tissues (Solheim 1995; Solheim & Krokene 1998). This may have allowed *G. clavigera* to initially grow more rapidly than *O. montium* in tree tissues.

Growth of *G. clavigera* would also be predicted to be faster than *O. montium* in trees during much of the summer at the study site based on average ambient temperatures.

Grosmannia clavigera grows faster than *O. montium* on artificial media at temperatures below 22°C; however, *O. montium* grows faster than *G. clavigera* at temperatures above 27°C (Six & Paine 1997; Solheim & Krokene 1998). The two fungi grow at similar rates on artificial media between 22 and 27°C, and growth of both species slows as temperatures approach 0°C. In 2003, average temperatures for the last week of June and for the months of July and August in the nearby town of Thompson Falls never exceeded 23.2°C (NOAA: <http://www.ncdc.noaa.gov>). Actual site temperatures were likely slightly cooler because the site was 725 m higher in elevation than Thompson Falls. In early-season attacked trees, the percent of phloem colonized by both fungi together increased between T2 in late August and T3 the following June, while phloem colonized by *O. montium* only decreased and phloem colonized by *G. clavigera* remained the same. Cooler average monthly temperatures in September and October (average monthly temperatures at Thompson Falls were 16.4 and 10.8°C, respectively) would have slowed growth of both fungi; however, *G. clavigera* still grows almost three times as fast as *O. montium* at 10°C (Solheim & Krokene 1998). This may have facilitated *G. clavigera*'s colonization of territory already occupied by *O. montium*, leading to a decrease in the amount of phloem colonized by *O. montium* only in early-season attacked trees in the early fall. Growth of *O. montium* may have been minimal during this time and thus any gains in territory may have been negligible. However, this interpretation requires some caution as only two early-season attacked trees were available for sampling at the site. In mid- and late-season attacked trees, the percent of phloem colonized by each fungus remained relatively constant between T2 and T3 (Figures 5b and c). Because sampling for T2 and T3 occurred later in the fall in mid- and late-season attacked trees (late

September and mid-October, respectively), temperatures were likely too cold for either fungus to grow substantially between these two sample times (average monthly temperatures fell below 0°C in November).

These results raise the question of whether *G. clavigera* and *O. montium* actually compete in the phloem of beetle-attacked trees. Although there is likely substantial overlap in the niches of the two fungi, competitive exclusion of one fungus by the other did not occur over the one year time period. Furthermore, hyphae of the two fungi were apparently intermingled in the phloem, although the relative density of hyphae or the biomass of each species was not discernable. These conclusions are consistent with my results from Chapter 2, which found that both *G. clavigera* and *O. montium* were able to colonize and coexist in artificial media already occupied by the other, although fungal growth slowed when the other species was encountered. Although I was unable to discern if fungal growth slowed when phloem colonized by the other species was encountered in this study, the lack of a negative correlation between the percent of phloem colonized by *G. clavigera* only and *O. montium* only (Table 1) indicates that strong interference (direct) competition, such as deadlock-, combative-, or replacement-type interactions, do not occur between mycelia of these two species. Interestingly, the percent of phloem colonized by *O. montium* only was significantly negatively correlated with the percent of phloem colonized by both fungi together at T2 and T3. This supports my previous assertion that *G. clavigera* colonized areas already occupied by *O. montium*, likely during the fall. The decline in the percent of phloem colonized by both fungi together at T4, and the corresponding increase in phloem yielding no fungi, suggests that both fungi were adversely affected when growing together. This may be due to an added strain on

resources, such as moisture (discussed below), which could be exacerbated when the two fungi co-occur. If a common, limiting resource is responsible for the decrease in phloem colonized by both fungi together and the corresponding increase in the percent of phloem yielding no isolations of fungi, then the results of this study indicate exploitation (indirect) competition between *G. clavigera* and *O. montium*.

The effects of phloem moisture on fungal growth were strongest at the beginning and end of the one year life cycle of the MPB. Attack by the MPB occurred during the last week in July and corresponded with decreasing phloem moisture (Figure 1). MPB either preferentially attacked, or were more likely to successfully attack, trees with lower levels of phloem moisture (Figure 2). Low phloem moisture may be indicative of water stress in trees as the xylem (water conducting tissue) and phloem are hydraulically connected (Hölttä et al. 2006). Other studies have also found that the MPB attacks trees in the summer after sapwood and phloem moisture levels have declined (Reid 1961). Trees of low vigor, such as those under water stress, have also been found to be less resistant to bark beetle and fungal colonization (e.g., Reid & Shrimpton 1971; Raffa & Berryman 1982; Miller et al. 1986; Croise & Lieutier 1993). Thus, beetles may preferentially attack water-stressed trees, or attacks on water-stressed trees may be more likely to succeed because of lowered tree defenses.

While it is widely accepted that the ability of water-stressed pines to physically ‘pitch out’ or expel attacking beetles is diminished, there may also be another benefit to the beetle of attacking such trees. Water-stressed trees may be a better medium for fungal growth allowing beetles to benefit from their association with fungi sooner. Fungal growth is inhibited in wood with a high moisture content (generally >120% dry weight)

and a low oxygen content; optimal moisture content for fungal growth in wood is between 60 and 80% (reviewed in Seifert 1993). Thus, fungal growth may initially be inhibited in newly attacked trees, especially those with high moisture contents. Negative correlations between phloem moisture and fungal growth at early sample times support this hypothesis, although the effect was greater for *O. montium* than *G. clavigera* (Table 2). The fungi may be able to colonize the phloem of trees with lower initial moisture levels more rapidly than trees with higher moisture levels. In fact, the fungi were able to colonize more phloem in late-season attacked trees compared to early- and mid-season attacked trees by T1 (Figure 4). Faster fungal colonization rates in late-season attacked trees may have been due to lower initial phloem moisture levels at the time of attack (<120% at T0) compared to early- and mid-season attacked trees (>140% at T0) (Figure 3). Attacking trees with lower phloem moisture would promote faster colonization of the phloem by fungi and allow the beetles to receive benefits (e.g., nutritional supplementation or phloem detoxification) from their fungal symbionts sooner.

While fungal growth may have initially been inhibited by excessive phloem moisture (and limited oxygen), fungal survival may have ultimately been reduced by a shortage of phloem moisture at the end of the one-year developmental period of the beetle. Moisture in wood becomes limiting for the growth and survival of ophiostomatoid fungi below 20% (Seifert 1993; Kim et al. 2005). My results indicate that moisture becomes limiting at the most critical time in the one-year life cycle of the beetle – when teneral adults are feeding and acquiring spores prior to emergence (Figure 3). The drop in the percent of phloem colonized by ophiostomatoid fungi between T3 and T4, and the corresponding increase in phloem with no live fungus, may be due to inadequate phloem moisture to

sustain the fungi (Figure 4). The overall drop in the percent of phloem colonized by ophiostomatoid fungi was a result of the decrease in phloem colonized by both fungi together and to a lesser extent of only *O. montium*; the percent of phloem colonized by only *G. clavigera* remained constant (Figure 5). Stronger positive correlations between phloem moisture and the percent of phloem colonized by both fungi together compared to each fungus alone late in the one year period also supports moisture being most limiting when the two fungi colonized phloem together (Table 2). Both species ultimately appear to be negatively affected when one species invades territory already occupied by the other. A potential explanation may be that water use is higher when the two fungi are growing together if the density of hyphae, or fungal biomass per unit area, increases when one fungus colonizes an area already occupied by the other species. Moisture may be the common limiting resource leading to strong indirect (exploitation) competition between the two fungi towards the end of the one year period.

Phloem moisture decreased rapidly in all trees once they were mass attacked by beetles (Figure 3); however, phloem moisture was not well-correlated between sample times (Table 2). This suggests that multiple factors likely interact over time to determine the drying process. Potential factors include relative humidity, temperature, aspect or degree of exposure of tree bole, rate of beetle development, rate of fungal colonization, and proportion of the tree bole attacked. Phloem was rapidly colonized by microorganisms after mass attack (Figures 4 and 5) and optimal moisture levels for fungal growth (60-80%, Seifert 1993) were reached by T2. Thus, conditions for optimal fungal growth are concurrent with the presence of late instar larvae, the life stages during which most of the total consumption and growth typically occurs in insects (Scriber &

Slansky 1981). In this study, as in others, *G. clavigera*, *O. montium*, and yeasts were the most common microorganisms isolated from tissues of lodgepole pine trees attacked by the MPB, indicating a close association among these organisms (Whitney 1971; Lee et al. 2006; Kim *et al.* 2005; Adams & Six 2007).

Our results indicate that while exploitation competition was prevalent when the two fungi colonized phloem together, interference competition was limited in this system. This may partly explain why competitive exclusion did not occur despite apparent substantial overlap in resource use, and why the two fungi are able to coexist while sharing a common resource. Interference competition, and even exploitation competition, competition will often result in the exclusion of one of the competitors. However, species engaging in exploitation competition may coexist if each species can maintain an exclusive area, or if there is some mechanism for partitioning the resource (Schoener 1976). Interestingly, both fungi maintained almost the same amount of exclusive territory at the most critical time – the period when fungal spores are acquired by beetles prior to dispersal - despite fluctuations in area occupied throughout the year (Figure 5). It is unknown if *G. clavigera* and *O. montium* partition resources in the phloem; however, they respond differently to potassium chloride compared to sucrose when the solutes are used to amend the water potential of media, suggesting that they may differ somewhat in their resource use or environmental tolerances (Chapter 2). Environmental variability may also promote coexistence of similar species by differentially affecting their growth rates and competitive abilities (e.g., Schoener 1976). Conditions in beetle-attacked trees change considerably over time and fungi may be differentially affected by water availability, temperature, tree defensive chemicals, and the presence of other

microorganism (Klepzig & Wilkens 1997; Lombardero et al. 2003; Klepzig et al. 2004; Hofstetter et al. 2005, 2006b, 2007; Adams & Six accepted; Chapter 2). Differences in the temperature and oxygen tolerances of the two fungi may also lead to niche differentiation and promote coexistence of the two species (Six & Paine 1997; Solheim & Krokene 1998; Six & Bentz 2007). In fact, it has been demonstrated that the relative abundance of fungal associates carried by bark beetles varies with temperature (Hofstetter et al. 2007; Six & Bentz 2007) as well as with population levels of phoretic mites and their fungal symbionts (Lombardero et al. 2003; Hofstetter et al. 2006b). Living in such a dynamic habitat, bark beetles may benefit from having multiple fungal symbionts that prosper under different environmental conditions (Hofstetter et al. 2007; Six and Bentz 2007). Resource partitioning, even on a fine scale, a constantly changing environment, and maintaining exclusive areas, may promote coexistence of species with overlapping niches, and prevent competitive exclusion from occurring before the organisms are transported to the next habitat.

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Table 1. Pearson's correlation coefficients (r) for comparisons of the percent of phloem colonized by either *G. clavigera* only, *O. montium* only, or both fungi growing together at four sample times in lodgepole pine trees attacked by the mountain pine beetle.

	T1 ¹	T2	T3	T4
<i>C. clavigera</i> only/ <i>O. montium</i> only	0.22	-0.25	-0.29	0.25
<i>G. clavigera</i> only/both fungi together	0.26	-0.29	-0.27	0.04
<i>O. montium</i> only/both fungi together	0.20	-0.69*	-0.70*	0.42

¹15 trees were sampled 4 times over the one year life cycle of the mountain pine beetle: T1, most brood were eggs or early instar larvae; T2, most brood were late instar larvae (prior to overwintering); T3, most brood were prepupae or pupae; T4, most brood were teneral adults.

* $P \leq 0.05$

Table 2. Pearson's correlation coefficients (r) for comparisons between the percent of phloem colonized by fungi at four sample times and phloem moisture at the same and preceding sample time in lodgepole pine trees attacked by the mountain pine beetle.

<i>G. clavigera</i> only						<i>O. montium</i> only					
Phloem moisture						Phloem moisture					
Colonized phloem	T0 ¹	T1	T2	T3	T4	Colonized phloem	T0	T1	T2	T3	T4
T1	-0.24	0.05				T1	-0.42	-0.50*			
T2		0.08	0.10			T2		-0.10	-0.29		
T3			-0.13	0.02		T3			-0.06	-0.24	
T4				-0.12	0.19	T4				0.27	0.28

<i>G. clavigera</i> & <i>O. montium</i> growing together						Total <i>G. clavigera</i> & <i>O. montium</i>					
Phloem moisture						Phloem moisture					
Colonized phloem	T0	T1	T2	T3	T4	Colonized phloem	T0	T1	T2	T3	T4
T1	-0.47	0.01				T1	-0.44	-0.20			
T2		-0.24	0.09			T2		-0.59*	-0.26		
T3			0.42	0.53		T3			0.49	0.46	
T4				0.71*	0.30	T4				0.62*	0.31

¹15 trees were sampled 4 times over the one year life cycle of the mountain pine beetle: T1, most brood were eggs or early instar larvae; T2, most brood were late instar larvae (prior to overwintering); T3, most brood were prepupae or pupae; T4, most brood were teneral adults.

* $P \leq 0.05$

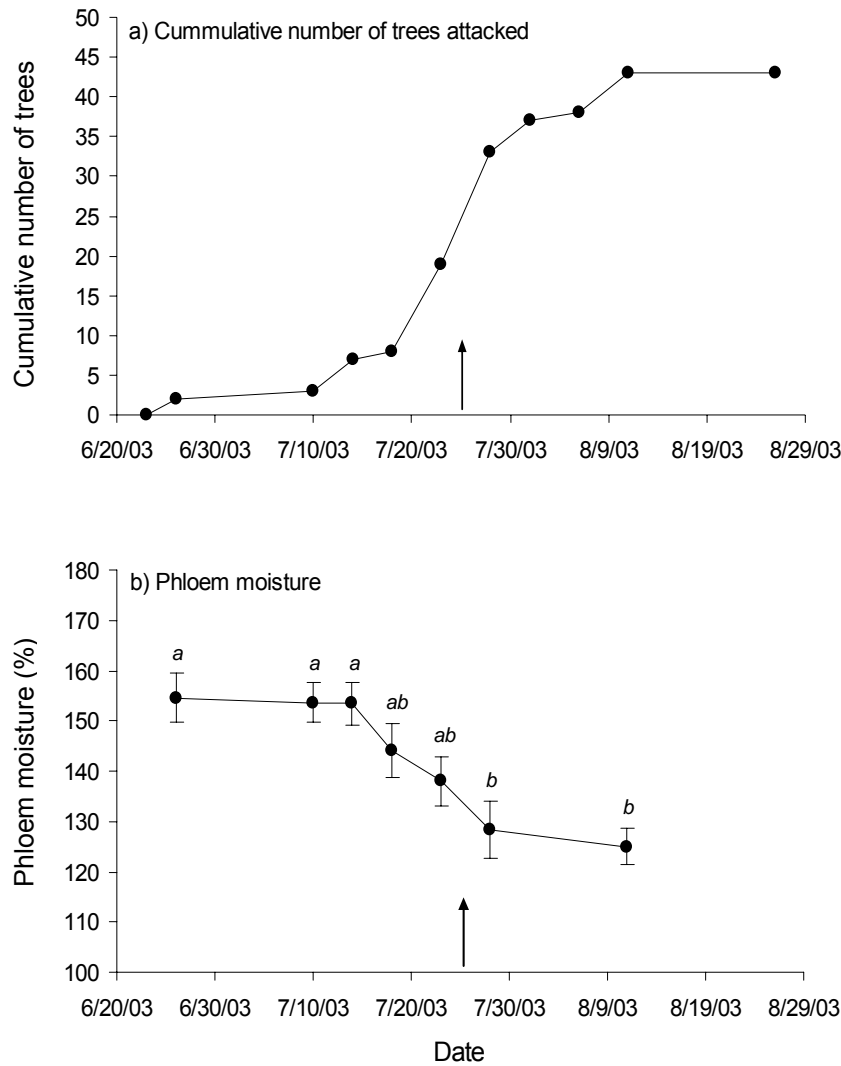


Figure 1. Effect of time on (a) the cumulative number of lodgepole pine trees mass attacked by the mountain pine beetle, and (b) phloem moisture (% dry weight). Arrows indicate peak attack time for the mountain pine beetle at the site. Phloem moisture was measured on non-attacked trees, but many of the sample trees were subsequently attacked by the mountain pine beetle and had to be replaced by new trees. As a result, phloem moisture measurements include 6 trees that were never attacked by the mountain pine beetle: 1 tree was sampled at 3 different sample times, 1 tree was sampled at 2 different sample times, and 4 trees were sampled at only one sample time. Ten trees were sampled either prior to being attacked or on the day they were attacked (T0, see text). Sample sizes (n) for phloem moisture at each date in the order they occur on the x-axis are 4, 7, 5, 3, 4, 3, and 8 trees. For phloem moisture, LS Means (\pm SE) are shown and dates with the same letter are not significantly different at $P \leq 0.05$.

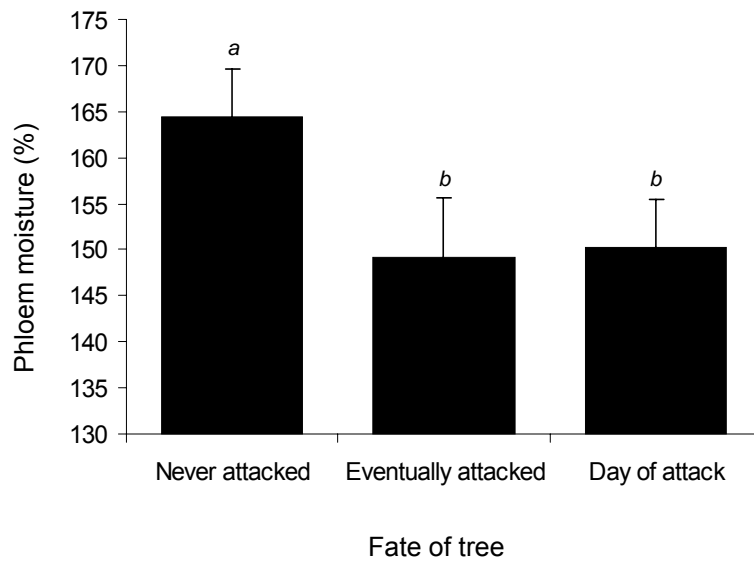


Figure 2. Phloem moisture (% dry weight) of lodgepole pine trees that were not attacked by the mountain pine beetle (never attacked), trees that were sampled prior to being attacked (eventually attacked), and trees that were sampled before and on the day of attack. Sample date was included as a factor in the analysis of variance because phloem moisture decreases in trees over the summer. The ‘never attacked’ category includes 9 measurements on 6 different trees: 1 tree was sampled at 3 different sample times, 1 tree was sampled at 2 different sample times, and 4 trees were sampled at only 1 sample time. Ten ‘eventually attacked’ and 15 ‘day of attack’ trees were sampled at one sample time each. LS Means (+SE) are shown so that variation due to sample date is removed. Trees classes with the same letter are not significantly different at $P \leq 0.05$.

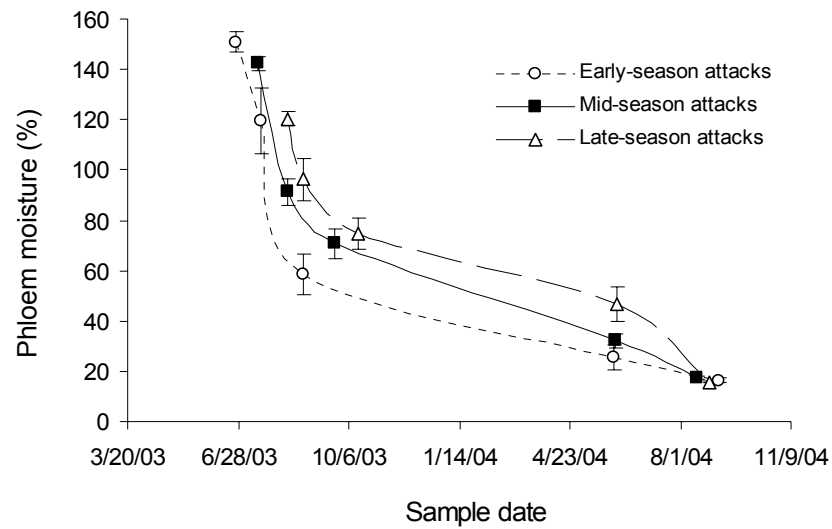


Figure 3. Phloem moisture (% dry weight) of lodgepole pine trees successfully mass attacked by the mountain pine beetle at different times in the summer: early-, mid-, and late-season. Phloem moisture was sampled 5 times over the one year life cycle of the beetle: the day of mass attack (T0); 2-4 wks after attack (T1, most brood were eggs or early instar larvae); 9-11 wks after attack prior to overwintering (T2, most brood were late instar larvae); 42-47 wks after attack (T3, most brood were prepupae or pupae); and 52-57 wks after attack prior to emergence (T4, most brood were teneral adults). For each type of attacked tree (early-, mid-, or late-attack), the symbols on the line correspond to T0 through T4.

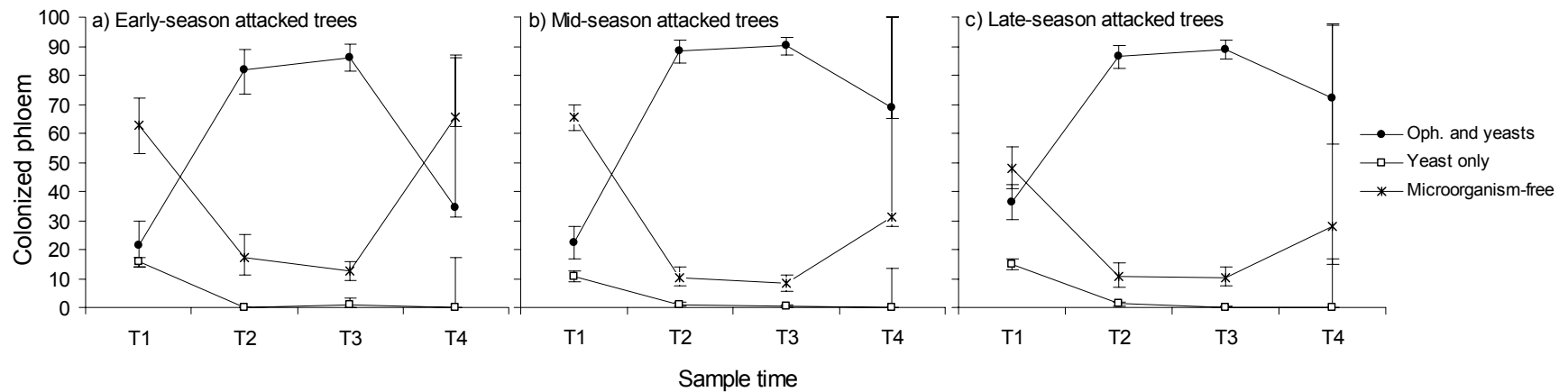


Figure 4. Percent of phloem colonized by ophiostomatoid fungi and yeasts (*G. clavigera* and *O. montium* alone or in combination), yeast only, or microorganism-free, in lodgepole pine trees attacked by the mountain pine beetle. Trees were attacked (a) early-, (b) mid-, or (c) late-season ($n=2, 8$, and 5 trees, respectively) and were sampled four times over the one year life cycle of the mountain pine beetle: T1, 2-4 wks after attack (most brood were eggs or early instar larvae); T2, 9-11 wks after attack prior to overwintering (most brood were late instar larvae); T3, 42-47 wks after attack (most brood were prepupae or pupae); and T4, 52-57 wks after attack prior to emergence (most brood were teneral adults). Back-transformed means (\pm SE) are shown.

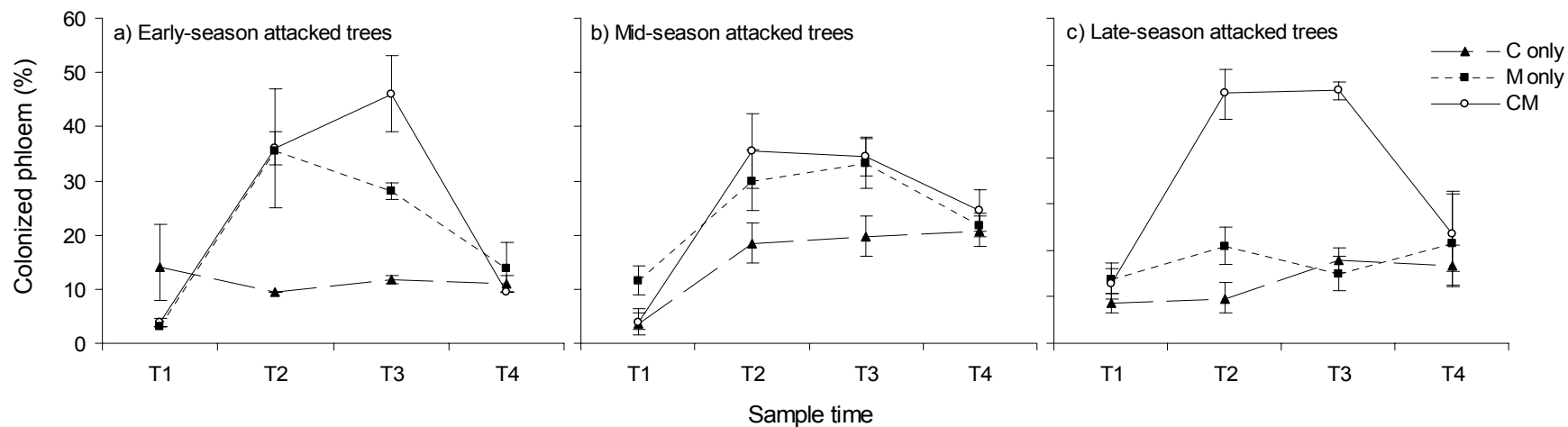


Figure 5. Phloem colonized by *G. clavigera*-only (C only), *O. montium*-only (M only), or both fungi (CM) growing together in the phloem of lodgepole pine trees attacked by the mountain pine beetle. Trees were attacked (a) early-, (b) mid-, or (c) late-season ($n=2$, 8, and 5 trees, respectively) and were sampled four times over the one year life cycle of the mountain pine beetle: T1, 2-4 wks after attack (most brood were eggs or early instar larvae); T2, 9-11 wks after attack prior to overwintering (most brood were late instar larvae); T3, 42-47 wks after attack (most brood were prepupae or pupae); and T4, 52-57 wks after attack prior to emergence (most brood were teneral adults). Back-transformed means (\pm SE) are shown.

Chapter 4

Transport of microbial symbionts by the mountain pine beetle, *Dendroctonus ponderosae*

Abstract

Grosmannia clavigera and *Ophiostoma montium* are both closely associated with the mountain pine beetle (MPB). The fungi may be transported on the body of the MPB or in maxillary (oral) mycangia (specialized structures of the integument for transporting fungal symbionts). Spores of the fungi likely become trapped in pits or under setae on the body; however, it is not known whether these or other structures consistently transport the fungi on the exoskeleton and thus also function as mycangia. I examined transport of *G. clavigera* and *O. montium* by the MPB using scanning electron microscopy and by isolating fungi from both mycangia and exoskeletons. Transport of the fungal associates on exoskeletons appears to be haphazard; neither fungus appeared to be highly adapted to transport on the body and structures on the exoskeleton (e.g., pits, asperites, setae) did not appear to be specialized for fungal transport. Based on isolation frequencies, there was no evidence of differential transport of *G. clavigera* and *O. montium* between the body and mycangia. Examination of exoskeletons using SEM also found no evidence that *G. clavigera* and *O. montium* are differentially transported on the body. Mycangia are large enough to accommodate all sizes of conidia produced by *O. montium* and some conidia produced by *G. clavigera*; however, conidia of either fungus were rarely observed at mycangial openings. The fungal material observed at some mycangial openings suggests that the fungi likely exist inside the mycangium in an altered, yeast, or yeast-like state.

Introduction

Ambrosia beetles and bark beetles are closely related (Coleoptera: Curculionidae, Scolytinae), but exploit different habitats: ambrosia beetles live in the sapwood of trees, while bark beetles live under the outer bark in the phloem of trees. However, associations with fungi are common in both types of beetles. Ambrosia beetles and their fungal symbionts provide a classic example of mutualism: the beetles receive nutritional benefits from feeding on the fungi which line their galleries and the fungi benefit from consistent transport to suitable habitats, which would otherwise be inaccessible (e.g., Francke-Grosmann 1963; Beaver 1989; Harrington 2005). Comparatively less is known about the relationships between bark beetles and their fungal associates (e.g., Francke-Grosmann 1963; Six 2003a; Harrington 2005). Evidence suggests that some species may receive dietary benefits from consuming phloem colonized by fungus or pre-emergence feeding on spores lining the pupal chambers (Whitney 1971; Fox et al. 1992; Coppedge et al. 1995; Six & Paine 1998; Ayres et al. 2000; Bleiker & Six in press). Additional benefits to the beetle may also include conditioning host tree tissues for brood development (e.g., promoting chemical and moisture changes), and aiding tree-killing bark beetles in overwhelming tree defenses (reviewed in Paine et al. 1997).

The benefits of a mutualistic relationship can only be reaped by the partners if they are able to maintain the continuity of their association from one generation to the next (Graham 1967). Specialized structures of the integument for transporting fungi, called mycangia (Batra 1963), have been found in many ambrosia beetles and some bark beetles. Mycangia vary greatly in their location on the beetle and in their form (Six 2003a). The most complex mycangia are sac- and tube-like invaginations of the

integument lined with glands. Glandular secretions are thought to maintain and support the growth of fungal propagules within the mycangium and to select against non-symbiotic fungi (Batra 1963; Barras & Perry 1971; Happ et al. 1971). Initially, only sacs or tubes lined with glands were considered to be mycangia. However, Six (2003a) argued that any structure that consistently transports fungus and maintains the association, including non-glandular structures, should be considered a mycangium. Under this definition, structures on the exoskeleton such as pits, shallow depressions, and pubescent areas that habitually harbor fungus, may also serve as mycangia.

The mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins) is an eruptive, tree-killing bark beetle that has paired sac mycangia (glandular status unknown) on the maxillary cardines of both sexes (Whitney & Farris 1970). Whitney & Farris (1970) observed columns of spores and hyphae extending from the mycangial openings of some beetles. They also cultured a number of microorganisms from the mycangia, including two ophiostomatoid fungi, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer, and Wingf. (previously *Ophiostoma clavigera*) and *Ophiostoma montium* (Rumbold) von Arx, which are the most common fungi isolated from the mycangia of the MPB (Whitney & Farris 1970; Six 2003b; Six & Bentz 2007; Bleiker & Six in press). Recently, Lee & Breuil (2005) described the ophiostomatoid fungus *Leptographium longiclavatum* sp. nov., which may also be closely associated with the MPB. Past studies may have identified *L. longiclavatum* as its close relative *G. clavigera* because both fungi are pleomorphic and there is significant overlap in the size and shape of their conidiophores and conidia (asexual spores) (Upadhyay 1981; Tsuneda & Hiratsuka 1984; Lee & Breuil 2005; Lee et al. 2006). In addition, *G. clavigera* tends to produce long,

clavate conidia, which are also characteristic of *L. longiclavatum*, in the pupal chambers of beetles in trees, but typically produces smaller, cylindrical, obclavate or roundish conidia in culture (Tsuneda & Hiratsuka 1984). However, to date, the frequency of *L. longiclavatum* has been very low in beetle populations that have been sampled (Lee & Breuil 2005; Kim et al. 2005 (= *Leptographium* sp.)).

The fungal associates of MPB, particularly *G. clavigera*, may play a role in overwhelming tree defenses and conditioning phloem for brood development (Reid 1961; Reid et al. 1967; Raffa & Berryman 1983; Yamaoka et al. 1995; Solheim 1995; Lee et al. 2006; Rice et al. 2007). The MPB may also receive nutritional benefits from its fungal associates, which could have important implications for beetle population dynamics (Six & Paine 1998; Bentz & Six 2006; Bleiker & Six in press). Although results among studies have varied, overall, *G. clavigera* appears to be more beneficial than *O. montium* to developing insects in terms of brood production, development time and adult size (Six & Paine 1998; Bleiker & Six in press). However, larval preference for phloem colonized by both fungi together over phloem colonized by one species alone indicates that the two fungi may actually provide complimentary benefits (Bleiker & Six in press).

In addition to being carried in the mycangia, both species of fungi are commonly isolated from the exoskeleton of MPB adults. However, Six (2003b) found that *G. clavigera* was more often isolated from the mycangia of MPB than from the exoskeleton, while the reverse was true for *O. montium*. Six & Paine (1998, 1999) hypothesized that *G. clavigera* may be more adapted to dispersal in mycangia because the beetle has a longer shared evolutionary history with this fungus than with *O. montium* and that *O. montium* may be an opportunistic “cheater” in the system with spores better adapted for

transport on the exoskeleton. Some bark beetles, e.g., *Ips typographus* L., *I. pini* (Say), *I. sexdentatus* Boerner, *Scolytus ventralis* LeConte, and *D. pseudotsugae* Hopk., consistently transport their fungal symbionts in pits on the head, pronotum, or elytra that arguably function as mycangia (Furniss et al. 1990, 1995; Leveux et al. 1989; Livingston & Berryman 1972; Lewinsohn et al. 1994). The MPB possesses well-defined pits on the elytra; however, it is not known whether they also function as mycangia.

I used scanning electron microscopy (SEM) to examine the exteriors and mycangial openings of MPBs to determine where the microbial symbionts, specifically *G. clavigera* and *O. montium*, are transported on adult beetles. The main aims of this study were to determine if there is differential transport of *G. clavigera* and *O. montium* on the body and in the maxillary mycangia of the MPB and whether pits on the body also function as mycangia.

Methods

The adult beetles used in this study came from two locations, one near Superior and the other near Butte, MT, (approximately 205 km apart). Adult beetles from Superior were collected from trees successfully mass attacked by the MPB in the summer of 2002. Trees were felled in February 2003, cut into short logs and placed into rearing cages with collection cups. Collection cups were emptied every 4 to 5 d until emergence ended and live beetles were stored in a sealed container with moist paper towels at 4°C for 3 to 10 d until they were dissected. The same process was used to collect adult beetles emerging from short logs cut from trees attacked by MPBs near Butte in the summers of 2004 and 2005. For the Butte population, logs were cut in July 2005 (2004 attacks) and July 2006

(2005 attacks), just prior to brood emergence and the beetle's main dispersal flight in the area. Collection cups were emptied every 2 to 4 d in 2005 and every 1 to 2 d in 2006 until emergence ended. Live beetles were stored as described above for up to 2 d before they were dissected.

In 2003, one maxilla was dissected from the head of each of 42 beetles from the Superior population and prepared for SEM using preparation method 1 described below. In 2005, the maxillae of 20 beetles were similarly dissected, and one maxilla and the body (including the severed head and the intact thorax and abdomen) were prepared for SEM also using preparation method 1 (described below). In 2006, one maxilla and one elytron were excised from 51 beetles and placed onto 2% malt extract agar (MEA) amended with 100 ppm cycloheximide (to reduce contamination by incidental fungi). Cultures were stored at 22°C for a minimum of 4 wk and fungi were identified using morphological characteristics (Upadyay 1981). Autoclaved pine twigs were added to all cultures and they were re-examined 4 wk later. Pine twigs were primarily added to facilitate the identification of *O. montium*, which sporulates readily on pine twigs, but not on MEA (*G. clavigera* sporulates readily on MEA). Twenty of the 51 beetles were randomly selected and the second maxilla and the body (including the head, thorax, and abdomen with one elytron) were prepared for SEM using preparation method 2 (described below).

To facilitate the identification of spores found in mycangial openings or on the exoskeleton using SEM, three isolates each of *G. clavigera* and *O. montium* were cultured on MEA and prepared for SEM using methods 1 or 3 (described below). The

fungi were isolated from the DeBorgia and Butte beetle populations as part of another study (Bleiker & Six in press).

All maxillae and beetle bodies, as well as portions of fungal cultures, were mounted on stubs with double-sided tape or silver paste and coated with gold-palladium. The samples from Superior (2003) were prepared and examined at the Microscope and Imaging Center at California State University (Hayward). All maxillae and beetle bodies from Butte (2005 and 2006) were dried in a Balzers 030 critical point dryer (BAL-TEC AG, Fürstetum, Liechtenstein), coated using a Pelco Model 3 sputter coater (Ted Pella Inc., Redding, CA), and examined in a Hitachi S-4700 field emission SEM (Hitachi Inc., Pleasanton, CA) in the EMtrix Lab at the University of Montana.

Preparation Method 1

Samples were fixed in 2.5% gluteraldehyde in 0.1 mol/L phosphate buffer and stored at 4°C until they were prepared for SEM as follows. Specimens were rinsed three times in phosphate buffer (~5 min each), dehydrated in a graded ethanol series (~10 min each), and dried in a critical point dryer.

Preparation Method 2

Samples were prepared as in method 1, except that following dehydration in the graded ethanol series, samples were dried using hexamethyldisilazane (HMDS) as described in method 3 below, instead of in a critical point dryer.

Preparation Method 3

Small blocks of fungal-colonized MEA containing abundant conidiophores on the agar surface were cut from cultures and vapor-fixed using a liquid film of 37% formaldehyde for 30 min and then vapor-fixed using a liquid film of 2% osmium

tetroxide for 60 min. The samples were then washed three times in phosphate buffer (~5 min each), dehydrated in a graded ethanol series (~10 min each), washed with 1:1 ethanol:HMDS (~10 min), and then washed twice in HMDS (~10 min each). Samples were washed for a third time in HMDS and held at 22°C until the HMDS had completely evaporated.

Two-tailed exact binomial tests with a hypothesized proportion of 0.5 (i.e., H_0 : no difference) were conducted to test for potential differences in the frequency of isolations on media between the maxillae and exoskeletons for *G. clavigera*, *O. montium*, other species of fungi, and no fungi. A one-tailed exact binomial test was used to test if *G. clavigera* was more likely to be isolated from the maxillae than *O. montium*, given the relative frequency with which the two fungi were isolated from the exoskeleton. Because the relative abundances of the two fungi varies among sites and over time (Six & Bentz 2007; Bleiker & Six in press), the proportion of *G. clavigera* isolated from the maxillae was tested against its probability of being isolated from the exoskeleton to account for its frequency in the beetle population. Thus, the hypothesized proportion used in the one-tailed test was the proportion of all the exoskeleton isolations with *G. clavigera* and/or *O. montium* that contained *G. clavigera* (27 out of 40 isolations, or 0.68; see Results) (i.e., H_0 : probability *G. clavigera* isolated from maxillae > 0.68). Binomial tests were conducted using SPSS 10.0 (SPSS Inc., Chicago, IL).

Results

Spore appearance

Figs. 1 and 2 show conidia (asexual spores) of *G. clavigera* and *O. montium*. Some spores became wrinkled or deflated from the fixation process, but they still retained their characteristic shapes (Figs. 1c and 2d). There was no discernable effect of preparation method on the appearance of the spores; one sample had both turgid and wrinkled conidia in different areas of the sample. Conidia of these fungi have been described elsewhere (Rumbold 1941; Robinson-Jeffery & Davidson 1968; Upadhyay 1981; Tsuneda & Hiratsuka 1984) and descriptions will not be repeated here.

Transport of symbionts

Beetles from Superior, 2003. Figs. 3 and 4 show the location of the mycangial opening on the maxillary cardine. Mycangial openings were visible on 25 of the 42 maxillae; muscle process or hemolymph obscured the mycangial openings on 17 maxillae. No evidence of fungi or other microorganisms were visible at 4 of the mycangial openings. However, spore-like objects, yeasts, or fungal-like growth were visible at 21 openings and often formed a condensed mass (Fig. 5). It was not possible to determine if the spore-like objects were *G. clavigera* or *O. montium*. This may have been due to the fact that the fungi are pleomorphic and can exist in mycangia in altered forms (e.g., yeast) (Batra 1963; Whitney & Farris 1970; Tsuneda & Hiratsuka 1984). Spores resembling ascospores (sexual spores) of *O. montium* were visible on the palp of one maxilla. An extracellular substance was often associated with the microorganisms at the mycangial openings (Figs. 6-7), but it was unclear as to whether the substance was produced by the beetle or by the fungi or other microorganisms. Strands resembling

hyphae or compact conidiophores were visible protruding from four mycangial openings (Figs. 9-12).

Beetles from Butte, 2005. Mycangial openings were visible on 11 of the 20 maxillae examined; nine openings were obscured by muscle processes or hemolymph. *G. clavigera*-like conidia were only observed at the opening of one mycangium (Figs. 16-17); no *O. montium*-like spores were associated with any of the mycangial openings. The opening of one mycangium was devoid of any microorganisms. Yeasts, yeast-like objects, or other microbes (possibly bacteria (bacilli and cocci)) were observed at 10 of the 11 mycangial openings. The yeasts or yeast-like objects were usually visible as individual cells at the mycangial opening (Fig. 3), but occasionally formed a more condensed mass (Figs. 13-15). One cardine broke during dissection of the maxilla, revealing the inside of the mycangium, which was filled with yeasts embedded in an extracellular matrix (Figs. 18-19). A comparison between the fungi visible at the mycangial opening and on the exoskeleton was not possible in 2005 because only one beetle had spores at the mycangial opening, and the body of this beetle was destroyed during the preparation process and was discarded.

The bodies of 19 of these beetles were examined. *G. clavigera*- or *O. montium*-like conidia were only found on the exoskeletons of seven beetles; five bodies carried conidia resembling both species (one of these beetles also carried *O. montium*-like ascospores, one beetle carried only *G. clavigera*-like conidia, and one beetle carried only *O. montium*-like conidia. None of the seven beetles with spores resembling *G. clavigera* or *O. montium* on the exoskeleton had identifiable spores at the mycangial openings. Conidia resembling *G. clavigera* and *O. montium* were found on all the body regions

(head, thorax and abdomen), but were most commonly found in pits on the elytra. Yeasts were found on the exoskeleton of all beetles, on all regions of the body, but were most commonly lodged at the base of setae and in pits on the elytra.

Beetles from Butte, 2006. In 2006, isolations were made on media from one maxilla and one elytron from each of 51 beetles. Cultures from six of the beetles were contaminated and discarded. Isolations from eight of the 45 maxillae yielded no fungi; however, ophiostomatoid fungi or yeasts were always isolated from elytra (Table 1). There were no differences in the probability with which *G. clavigera* was isolated from maxillae and exoskeletons ($P=0.57$), or with which *O. montium* was isolated from maxillae and exoskeletons ($P=0.09$). Similarly, there was no difference in the probability with which other species of fungi (all yeasts) were isolated from maxillae and exoskeletons ($P=1.0$); however, maxillae were more likely to be devoid of fungi than exoskeletons ($P=0.008$). *G. clavigera* was no more likely than *O. montium* to be isolated from the maxillae given the isolation frequency of the two fungi from the elytra ($P=0.21$).

The mycangial openings on 15 of the 20 maxillae examined in 2006 were visible using SEM. Yeasts were visible at five of the mycangial openings, and *G. clavigera*, *O. montium*, or other fungi were isolated on media from the second maxillae taken from all 5 of these beetles. The yeasts in some of these openings may have been *G. clavigera* or *O. montium* in yeast form (Figs. 13-15). Ten mycangial openings appeared to be devoid of fungi, although microbial growth or extracellular material was often present (Figs. 20 and 21) and may have actually obscured spores in some cases. *G. clavigera*, *O. montium*, or other fungi were isolated on media from the second maxillae taken from seven of these 10 beetles (Table 1).

The bodies of 16 beetles were examined in 2006 using SEM; four bodies were discarded because much of the elytron was covered by what appeared to be tree resin. Both *G. clavigera*-like conidia and *O. montium*-like conidia were visible on the exoskeletons of eight beetles (Figs. 22-24, 26 and 27-29). Ascospores resembling those of *O. montium* were also present on one of these beetles. Examination of the bodies of the eight beetles from which both fungi were isolated from the excised elytron found *G. clavigera*-like conidia on one beetle and *O. montium*-like conidia on five beetles; no ophiostomatoid fungal spores were visible on two of the beetles. Another two beetles also carried only *G. clavigera*-like conidia on the body, and cultures from the excised elytra from these beetles yielded only *G. clavigera*. Similarly, another two beetles carried only *O. montium*-like conidia on the body and cultures from the excised elytra from these beetles yielded only *O. montium*. No *G. clavigera*- or *O. montium*-like spores were visible using the SEM on four exoskeletons; however, *O. montium* was isolated from the excised elytron from all four of these beetles, and *G. clavigera* was isolated from two of these beetles. Similar to 2005, spores resembling both species of fungi were found on all body regions. It was not uncommon for the majority of pits on the exoskeleton of beetles to be relatively empty and contain no spore-like objects (Fig. 25). In general, the total number of spores of either species of fungus on the exoskeleton was low on the majority of beetles. A crust, formed from what appeared be very small conjoined spheres, was often found in association with objects, including spores, on the exoskeleton and may have obscured the identity of some spores. Similar to 2005, yeasts were visible on the exoskeleton of all beetles examined and were most abundant at the base of setae and in pits.

Examination of one maxilla and the body (minus one elytron) of 15 beetles using SEM found that spores resembling *G. clavigera* or *O. montium* were rarely observed in mycangial openings; however, spores resembling at least one of the fungi were found on most of the bodies examined (Table 2).

Size of mycangial opening

The mycangial opening is oblong in shape (Figs. 3, 4, 16 and 20). The opening follows the contour of the cardine giving it a general “U” shape. This U-shape confounds the measurement of its length. However, in general, the length of the mycangial opening was approximately 26 to 39 μm long with the average length being 33 μm . The internal dimensions of the mycangia of the MPB are unknown; however, the mycangium that broke open was 37 μm across in the plane in which it broke (Fig. 18). The mycangial opening was slightly constricted at its center and ranged from 3.0 to 5.5 μm wide (average 4.6 μm) at its center.

Discussion

The MPB is able to transport both *G. clavigera* and *O. montium* in its maxillary mycangia as well as on the exoskeleton. Spores on the exoskeleton were found on the head, thorax and abdomen of the MPB, but were most common in pits and depressions on the elytra. Usually only small groups of spores were found on the exoskeleton of MPBs and the pits were never as full of spores as has been observed with bark beetle species that lack sac- or tube-type mycangia and rely solely on pit mycangia to transport their fungal symbionts, e.g., *I. typographus*, *I. pini*, *I. sexdentatus*, *S. ventralis*, and *D. pseudotsugae* (Furniss et al. 1990, 1995; Levieux et al. 1989; Livingston & Berryman

1972; Lewinsohn et al. 1994). Spores may have been washed off beetles in the current study during sample preparation; however, Lewinsohn et al. (1994) used a preparation method similar to method 1 in this study and still found numerous spores in pits on the exoskeleton of *D. pseudotsugae*. The method of collecting the beetles may also have affected the spore load. Pre-flight *D. pseudotsugae* removed from under the bark carried more spores than parent beetles removed from egg galleries (Lewinsohn et al. 1994). Six (2003b) isolated *G. clavigera* more frequently from the exoskeletons of pre-flight MPBs removed from under the bark than from the exoskeletons of beetles caught in flight traps. The MPBs examined in this study were collected shortly after they emerged from logs and thus would be expected to have a high spore load because beetles acquire spores in the pupal chamber just prior to emergence. Pits, asperites, and setae can trap spores, but I found no evidence that any of these structures consistently carried fungi and functioned as true mycangia for the MPB. In addition, I found no evidence of an oily or waxy substance in the pits, which is thought to protect spores from desiccation, as has been associated with spores in pit-type mycangia on *I. pini*, *S. ventralis*, and *D. pseudotsugae* (Livingston & Berryman 1972; Lewinsohn et al. 1994). The results of this study indicate that fungal transport on the exoskeleton of MPB may be merely incidental, likely due to circumstance rather than adaptation.

Even though transport of the fungal associates on the exoskeleton of MPB appears to be haphazard, fungi are consistently isolated from the exoskeleton of adult beetles (Six 2003b; Lee et al. 2006; Bleiker & Six, unpublished data). Based on isolation frequencies, there was no evidence of differential transport of *G. clavigera* and *O. montium* between the body and mycangia. Examination of exoskeletons using SEM also found no evidence

that *G. clavigera* and *O. montium* are differentially transported on the body. Six (2003b) isolated fungi from the exoskeletons and mycangia of two groups of MPBs: pre-flight beetles removed from under the bark just prior to emergence and in-flight beetles collected in pheromone-baited traps. There was no difference in the isolation frequencies of *G. clavigera* and *O. montium* between exoskeletons and mycangia of pre-flight beetles removed from under the bark. However, for beetles caught in flight traps, *G. clavigera* was isolated more frequently from mycangia than exoskeletons, while the reverse was true for *O. montium* (Six 2003b). Spores of the two species may differ in their ability to survive on the exoskeleton (e.g., adhere to the exoskeleton, survive desiccation or ultra-violet exposure), and thus, differential transport of the two fungi on the body may only manifest after a significant flight period (Six 2003b). The beetles used in the current study had a minimal flight period because they were collected from rearing cages, which may explain why my results were similar to those of Six (2003b) for pre-flight adults removed from under the bark.

The size of the mycangial opening and the fortuitous view of the inside of a mycangium on the broken cardine indicate that mycangia are large enough to accommodate all sizes of conidia produced by *O. montium* and some conidia produced by *G. clavigera*. *O. montium* conidia range in size from 1.5 to 8 μm in length, while *G. clavigera* conidia range in size from 2 to 85 μm (Rumbold 1941; Robinson-Jeffery & Davidson 1968; Upadhyay 1981; Tsuneda & Hiratsuka 1984). Indeed, both *G. clavigera* and *O. montium* were commonly isolated from mycangia (in 2006), but there was no indication that one fungus was more likely to be carried in mycangia over the other given their relative abundances on the exoskeleton. Based on physical size, it should be easier

for conidia of *O. montium* to enter the mycangia than *G. clavigera*, especially since the latter species typically produces its longer-type conidia in pupal chambers (shorter conidia are more typically produced in culture) (Tsuneda & Hiratsuka 1984). Thus, any selection for *G. clavigera* over *O. montium* in the mycangia as proposed by Six (2003b) would likely be based on the environment inside the mycangia instead of physical factors. Evidence suggests that glandular secretions may select against non-symbiotic, ubiquitous fungi and promote growth of symbiotic fungi (Batra 1963; Barras & Perry 1971, 1972; Happ et al. 1971); however, it remains to be investigated whether secretions could select between, or differentially promote, two closely related ophiostomatoid species.

Despite frequent isolations of *G. clavigera* and *O. montium* from mycangia, conidia of either species were only rarely observed at mycangial openings using SEM (conducted in 2006). However, fungal material in the form of yeasts, yeast-like cells, hyphae-like strands or compact conidiophore-like structures were observed at the majority of mycangial openings examined on beetles collected in 2003 (Superior) and 2005 (Butte), and at one third of the openings from beetles collected in 2006 (Butte). It is likely that some of this fungal material was *G. clavigera* or *O. montium*. Whitney & Farris (1970) suggested that the fungal associates of MPB likely undergo change in the mycangium. Indeed, fungal associates of many ambrosia and bark beetles are known to multiply within the mycangium and to exist as yeast, or in a yeast-like state, which also may confound their identification based on morphology (e.g., Batra 1963; Abrahamson et al. 1967; Barras & Perry 1971, 1972; Barras & Taylor 1973; Happ et al. 1976; Paine & Birch 1983). The quantity of spores acquired in the mycangium by MPB during pre-emergence feeding and their growth form within the mycangium is unknown. If the MPB

only acquires a few conidia in its mycangium that then reproduce by budding, as is the case for the western pine beetle *Dendroctonus brevicomis* LeConte (Paine & Birch 1983), this could explain why a higher percentage of the mycangial openings on beetles collected in 2006 appeared to be empty. These beetles were collected and dissected within a shorter time frame than beetles collected in 2003 and 2005, so any fungal propagules acquired in the mycangia may not have had time to reproduce and fill the opening. Both *G. clavigera* and *O. montium* are capable of reproduction by budding in yeast form and are more likely to do so under certain environmental and nutrient conditions (Upadhyay 1981; Tsuneda & Hiratsuka 1984), which could exist within the mycangium. Reproducing by budding would also facilitate the transport of *G. clavigera* in the mycangium, which has longer conidia than *O. montium*. Smaller propagules produced by budding would more easily fit in and fill the mycangium. Based on the growth form of the objects visible at mycangial openings and the fact that *G. clavigera* or *O. montium* are usually isolated from the mycangia, it seems likely that these fungi exist in the mycangium in an altered state capable of reproduction by budding and that spores may even germinate within the mycangium as evidenced by hyphal-like strands and compact conidiophore-like structures (Figs. 8-15).

In addition to *G. clavigera* and *O. montium*, objects resembling yeasts, hat-shaped yeast ascospores (Figs. 28-29), and bacteria (bacilli and cocci) were present at some mycangial openings and on exoskeletons. Yeasts and bacteria are well known associates of bark beetles (e.g., Shifrine & Phaff 1956; Callaham & Shifrine 1960; Whitney & Farris 1970; Whitney 1971; Bridges et al. 1984; Levieux et al. 1989; Lewinsohn et al. 1994; Furniss et al. 1995; Adams & Six 2007); however, comparatively less is known

about their role in the system compared to *G. clavigera* and *O. montium*. Yeasts and bacteria may be involved in the synthesis of beetle pheromones (Brand et al. 1975), digestion of tree tissues (Delalibera et al. 2005), nitrogen fixation (Bridges 1981), insect mortality (Moore 1972), and have significant interactions with the ophiostomatoid fungal associates of bark beetles (Cardoza et al. 2006; Adams & Six accepted). Very small spheres that sometimes formed a solid crust, were present on the exoskeletons of beetles and maxillae, as well as cultures of fungi growing on media (Figs. 1c and 2d), that were prepared for SEM using methods 2 and 3. The spheres could be bacteria capable of forming a biofilm; however, no spheres were observed on the beetle or fungal samples prepared using method 1, so they may also be an artifact of the preparation process.

Mycangia have evolved independently several times in bark beetles (Six 2003a), which suggests that mycangia play an important role in maintaining the continuity of the bark beetle-fungal association from one generation to the next. The results of this study indicate that although MPB commonly carries conidia of *G. clavigera* and *O. montium* on the body, there are far less conidia on the exoskeleton compared to other bark beetle species that rely solely on exoskeletal transport of their fungal symbionts. Although ascospores may be transported by the MPB, conidia were the dominant spore type associated with beetle exoskeletons examined in this study. Conidia are the most common spore type found lining the pupal chamber, although ascospores may also be present. MPB acquires conidia in the pupal chamber, which likely then reproduce in a yeast or yeast-like state in the mycangium. In addition, spores may apparently germinate in the mycangium and produce hyphae, although this occurs less frequently. The yeast-like cells and hyphae exuding from the mycangia inoculate the next host tree as the

parent beetles construct their egg gallery. It seems that MPB could simply acquire enough conidia from pupal chambers to inoculate the next host tree and forgo any energy costs associated with maintaining and supporting fungal reproduction within the mycangium. However, egg gallery construction extends over a period of weeks, with the female continually extending the gallery into fresh, uncolonized phloem. Thus, the reproducing, yeast-like state of the fungi in the mycangium may provide a continual (regenerating) supply of inocula over an extended period of time for the parent beetles, and increase the rate at which tree tissues are colonized by the fungal symbionts.

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Table 1: The number of times *G. clavigera*, *O. montium*, both fungi, other species of fungi, or no fungi were isolated from the maxillae and elytra of mountain pine beetle adults from Butte (2006), MT.

Maxilla	Elytron				No fungi	Total
	C	M	C & M	Other fungi [†]		
C	9	2	3	3	0	17
M	4	3	2	0	0	9
C & M	0	2	3	0	0	5
Other fungi [†]	0	3	2	1	0	6
No fungi	2	3	2	1	0	8
Total	15	13	12	5	0	

C, *G. clavigera*; M, *O. montium*; C & M, both *G. clavigera* & *O. montium*.

[†] All of these cultures contained yeasts, but no filamentous fungi.

Table 2: The number of times spores resembling *G. clavigera*, *O. montium*, other species of fungi, or no fungal spores, were visible in mycangial openings and on elytra of mountain pine beetle adults from Butte (2006), MT, using scanning electron microscopy.

Maxilla	Elytron				No fungi	Total
	C	M	C & M	Other fungi [†]		
C	0	0	0	0	0	0
M	0	0	0	0	0	0
C & M	0	0	0	0	0	0
Other fungi [†]	0	1	1	1	0	3
No fungi	1	1	9	1	0	12
Total	1	2	10	2	0	

C, *G. clavigera*; M, *O. montium*; C & M, both *G. clavigera* & *O. montium*.

[†] All yeasts.

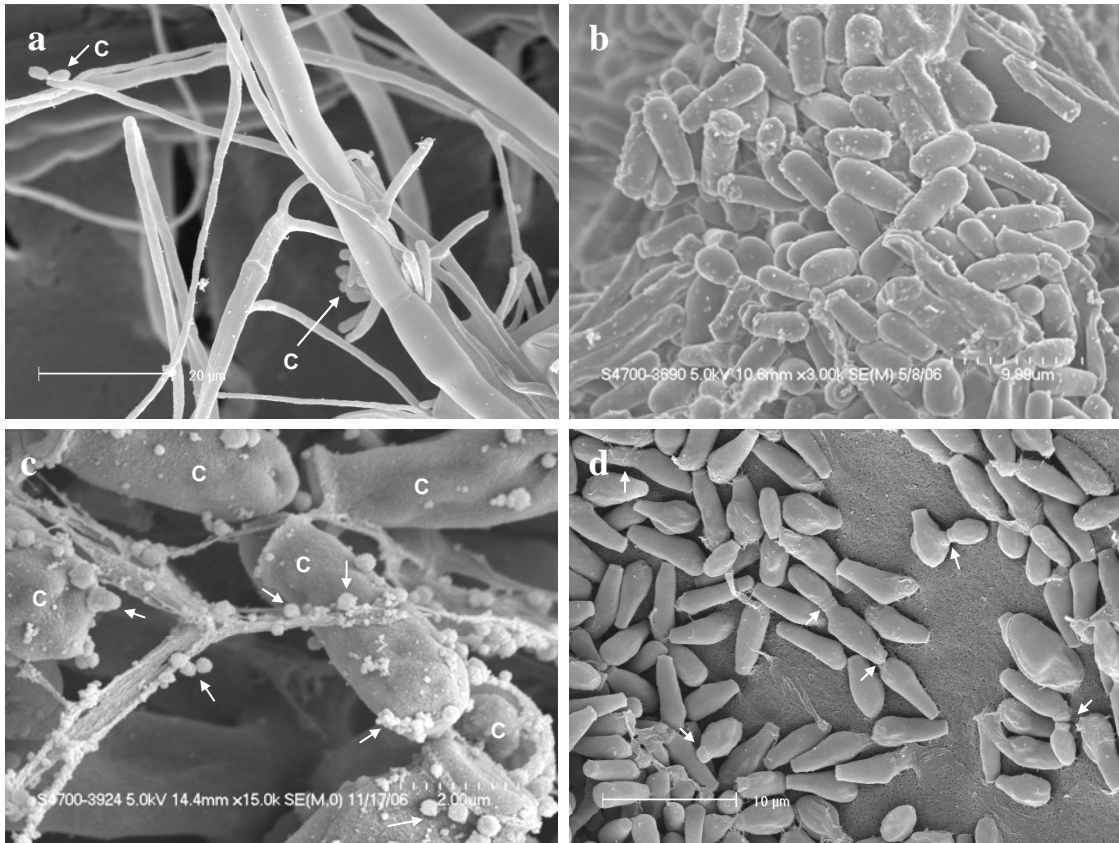


Figure 1. Scanning electron micrographs of *Grosmannia clavigera* growing on malt extract agar. (a) Hyphae and conidia (C), (b) a mass of conidia, (c) close-up of conidia (C), unidentified spheres (arrows) that may be bacteria (cocci) or an artefact of processing, and (d) budding conidia (arrows).

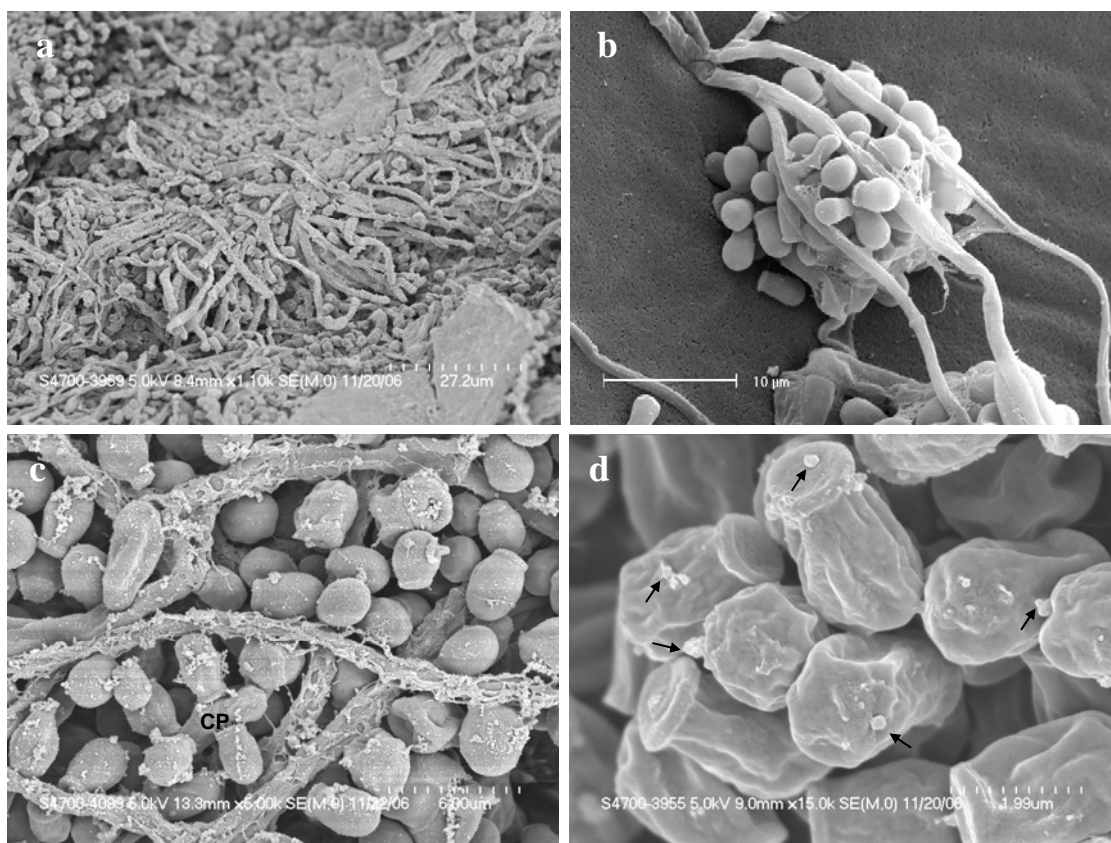
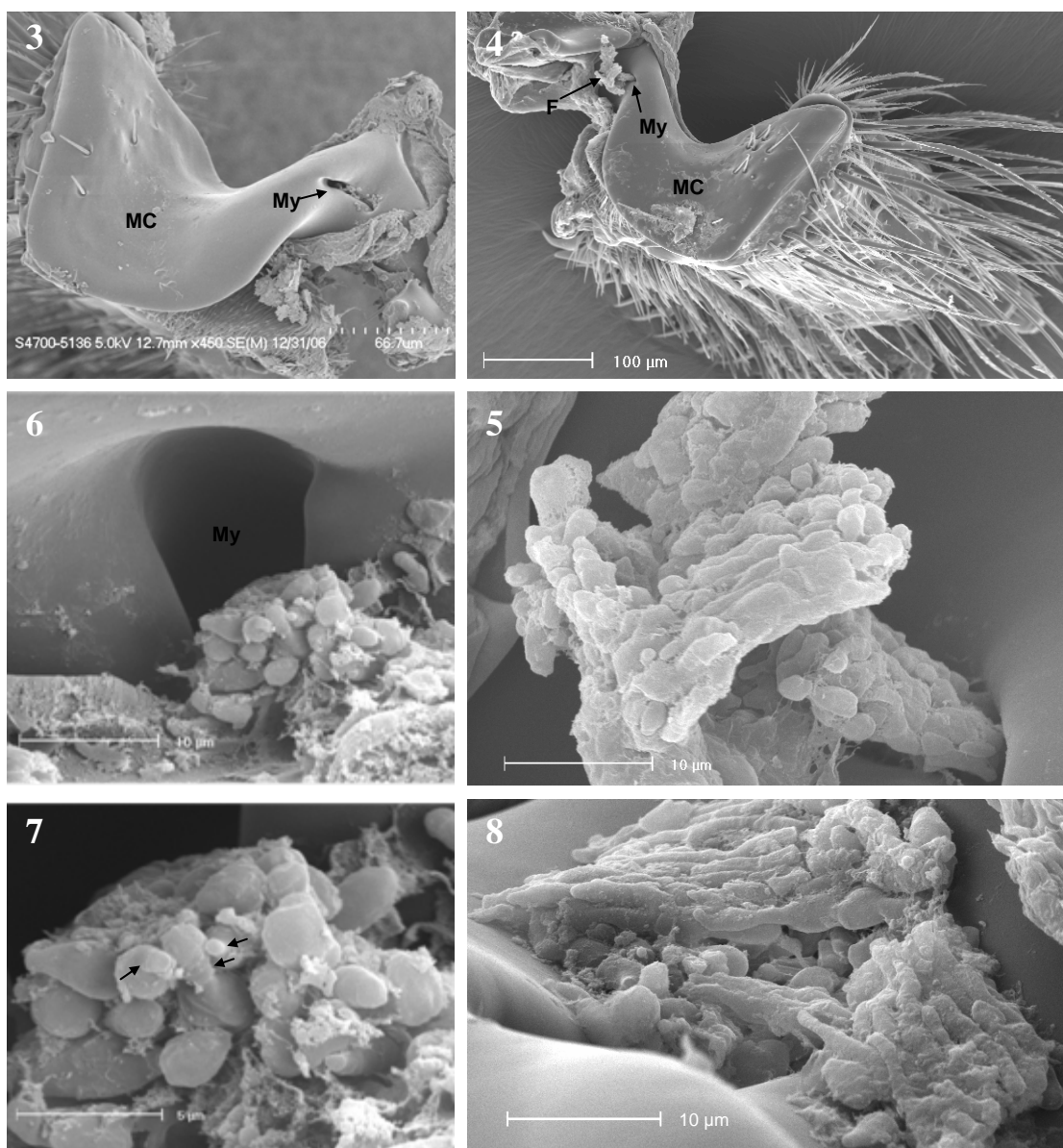
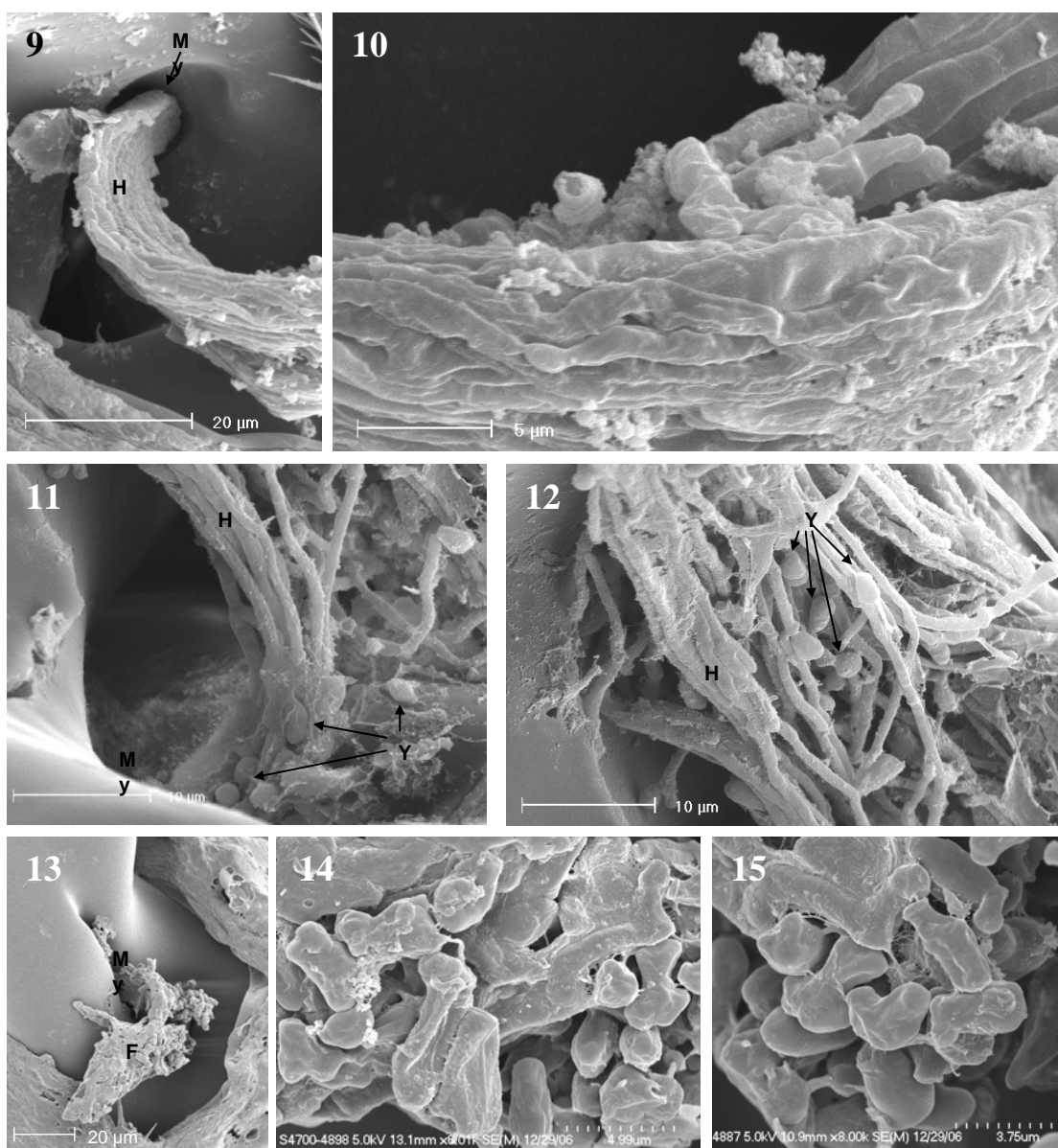


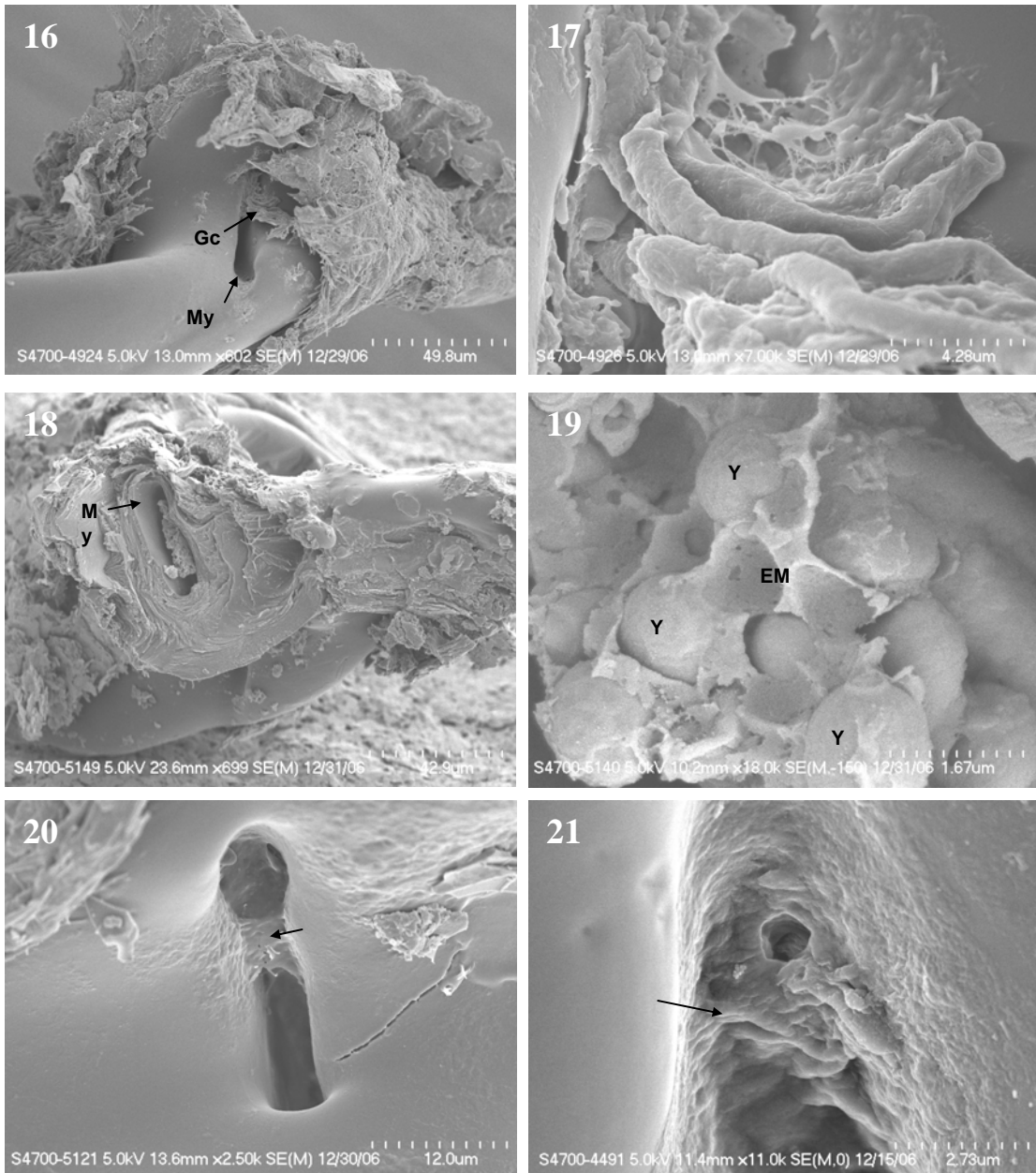
Figure 2. Scanning electron micrographs of *Ophiostoma montium* growing on malt extract agar. (a) Hyphae and conidia, (b) close-up of hyphae and conidia, (c) hyphae, conidia and the tip of a conidiophore (CP), (d) close-up of conidia (wrinkling is a result of processing) and unidentified spheres (arrows) that may be bacteria or an artefact of sample processing.



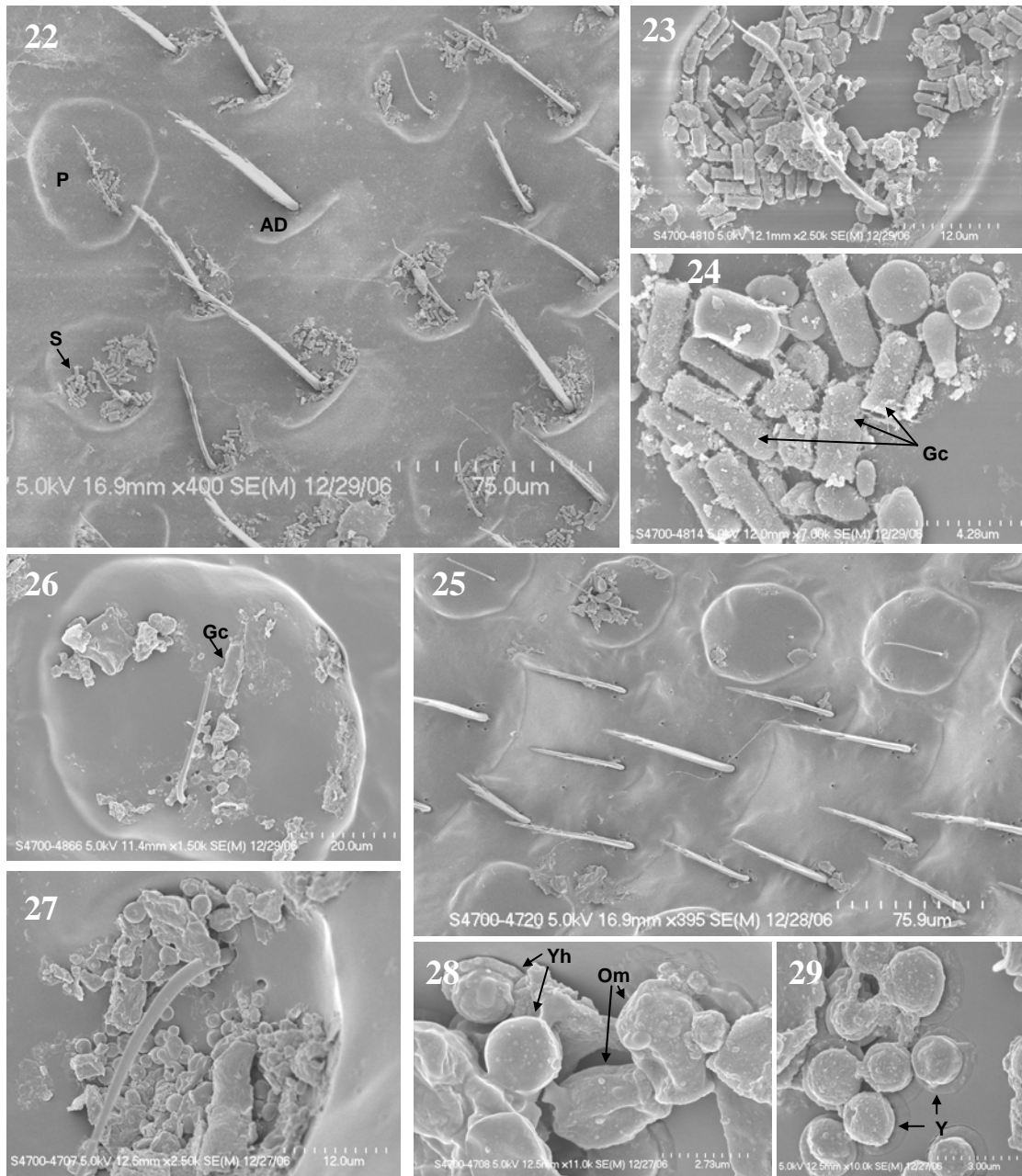
Figures 3-8. Scanning electron micrographs of mycangial openings on *Dendroctonus ponderosae*. Fig. 3. Mycangial opening (My) on the maxillary cardine (MC). Yeast-like cells visible around the mycangial opening. Fig. 4. Fungal material (F) protruding from mycangial opening. Fig. 5. Close-up of yeast-like fungal material in Fig. 4. Fig. 6. Yeast-like cells protruding from mycangial opening. Fig. 7. Close-up of Fig. 6 showing budding conidia (arrows). Fig. 8. Fungal material outside mycangial opening.



Figs. 9-15. Scanning electron micrographs of mycangial openings on *Dendroctonus ponderosae*. Fig. 9. Hypha- or conidiophore-like strands (H) extending from mycangial opening (M). Fig. 10. Close-up of hypha-like strands in Fig. 9. Figs. 11-12. Hypha-like strands and yeast-like cells (Y) at mycangial opening. Fig. 13. Fungal material (F) protruding from mycangial opening. Figs. 14-15. Close-ups of fungal material from Fig. 13 showing yeast or yeast-like growth protruding from mycangium.



Figs. 16-21. Scanning electron micrographs of mycangia of *Dendroctonus ponderosae*. Fig. 16. *Grosmannia clavigera*-like conidia (Gc) at mycangial opening (My). Fig. 17. Close-up of *G. clavigera*-like conidia in Fig. 16. Fig. 18. Broken end of maxillary cardine revealing inside of mycangium. Fig. 19. Close-up of Fig. 18 showing yeast-like cells (Y) embedded in extracellular matrix (EM) inside mycangium. Fig. 20-21. Extracellular material or microbial growth (arrow) at mycangial openings.



Figures 22-29. Scanning electron micrographs of *Dendroctonus ponderosae* elytra. Fig. 22. An elytron with spores (S) in pits (P) and depressions associated with asperites (AD). Figs. 23-24. Close-ups of spores from Fig. 22 showing *Grosmannia clavigera*-like conidia (Gc). Fig. 25. An elytron carrying few spores. Fig. 26. An elytral pit with one *G. clavigera*-like conidium. Fig. 27. An elytral pit with numerous spore-like objects. Figs. 28-29. Close-ups of Fig. 27 showing *Ophiostoma montium*-like conidia (Om), hat-shaped ascospores resembling yeasts (Yh), and other yeast-like cells (Y).